IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	
In Re Application of: Stanford Mark Moran	Confirmation No. 8022
Serial No.: 10/004,118	Art Unit: 1647
Filing Date: 30 October 2001	Examiner: Seharaseyon, J.
Title: METHOD FOR TREATING DISEASES WITH OMEGA INTERFERON	

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Thomas R. Alessi, hereby declare as follows:

2. I received my Bachelors of Science Degree in Chemistry from Allegheny College in 1980, my Master of Science Degree in Organic Chemistry from the University of Rochester in 1982, and my Doctorate of Philosophy Degree in Organic Chemistry from the University of Rochester in 1986.

3. I am currently Vice President, Development and Manufacturing, at Intarcia Therapeutics, Inc., and have held this position since August of 2007. My previous positions at Intarcia Therapeutics, Inc. (formerly BioMedicines, Inc.), were Vice President, Development, from February 2002 – August 2007, and Executive Director, Regulatory Affairs and Product Development from December 1997 - January 2002. I was previously employed by Schering-Plough Research Institute, Kenilworth, New Jersey as Senior Manager, U.S. Regulatory Affairs, from April 1992 - August 1993, and as Manager, U.S. Regulatory Affairs, from May 1990 - April 1992. During this time I worked on therapeutic applications of INTRON® A (Schering Corporation, Kenilworth, NJ) (interferon alfa-2b), including treatment of Hepatitis C Virus (HCV) infection using interferon alfa-2b. Additional details regarding my background and qualifications can be found in the accompanying copy of my *Curriculum Vitae* (*see* Appendix A).

4. At the time the present application, USSN 10/004,118 and the provisional application (USSN 60/245,883) from which the present application claims the benefit of priority, were filed, I worked at Intarcia Therapeutics, Inc. (formerly BioMedicines, Inc.). I worked on therapeutic applications of omega interferon at that time and am continuing to work on development and product manufacturing related to omega interferon at present.

5. I have reviewed pending U.S. Patent Application Serial No. 10/004,118 for "METHOD FOR TREATING DISEASES WITH OMEGA INTERFERON " by Stanford Mark Moran (hereinafter "the specification") including pending claims 87, 88, 90-96 and 98-114. I have also reviewed (1) the Office Action, mailed 28 May 2008; (2) Parker, et al., WO 00/40273; (3) Goeddel, et al., US 5,120,832; (4) Theeuwes, et al., US 4,976,966; and (5) Guillen, et al., US 6,074,673. Therefore, I am familiar with the issues raised by the Examiner. The reference of Theeuwes, et al., does not teach any method for treatment of HCV nor does the reference contain any teaching regarding interferon, specifically omega interferon. The reference of Guillen, et al., does not teach any method for treatment of HCV nor does the reference contain any teaching regarding interferon, specifically omega interferon. Accordingly, I will not be discussing the references of Theeuwes, et al., or Guillen, et al.

6. I understand that pending claims 87, 88, 90-96, 98-107, and 114 are directed to methods of treating HCV infection in a subject in need of such treatment, comprising administering a therapeutically effective amount of omega interferon protein to the subject. Further, I understand that these HCV treatment method claims relate to the administration of omega interferon protein either (i) at a controlled rate over time where the therapeutically effective amount of omega interferon is an amount of omega interferon selected from the group consisting of between 48 and 194 micrograms per week, between 23 and 388 micrograms per week, or between 23 and 623 micrograms per week, or (ii) by injection and the therapeutically effective amount of omega interferon is an amount of omega interferon selected from the group consisting of between about 1 and about 210 micrograms per week and between about 22.5 and about 360 micrograms per week. The only independent claims are claims 87, 88 and 114. All other pending claims ultimately depend from one of these claims and thereby incorporate all of the limitations of the independent claims from which

they depend.

7. In September of 2000, when the provisional application was filed from which the present specification claims the benefit of priority, a typical investigator working in the field of HCV treatment had a Ph.D. in the Biological or Chemical Sciences or an M.D., and two to five years of relevant experience post-degree. I will call such a person a "typical scientist."

8. In the Office action, mailed 28 May 2008, the Examiner asserts an argument against the patentability of the present claims based on two assumptions:

(i) that the reference of Parker, et al., "teaches HCV infection can be treated by IFN-ω [omega interferon]" (*see, e.g.,* Office action, mailed 28 May 2008, page 4); and

(ii) that the reference of Goeddel, et al., "teaches IFN-ω proteins with biological activities, such as antiviral activity, that are similar to the IFN-α that is also used to treat HCV" (*see, e.g.,* Office action, mailed 28 May 2008, page 4).

I will address these two arguments asserted by the Examiner in turn.

9. First, I am familiar with the teachings of the reference of Parker, et al. The reference asserts to provide a method of treating a viral disease comprising administering to a mammal a polynucleotide construct comprising a polynucleotide encoding omega interferon (*see* Abstract). Accordingly, this is a proposed gene therapy method. However, the only data related to treatment of a viral disease using such constructs is an *in vitro* example for anti-viral activity against Murine Encephalomyocarditis Virus (EMCV) in human (A549) and murine (L929) cells. The reference asserts that a polynucleotide encoding omega interferon might be used to treat HCV; however, there is no evidence or enablement to support this assertion. EMCV is in the viral family *Picornaviridae* whereas HCV is in the viral family *Flaviviridae*. No scientific basis has been presented to validate an extrapolation of the teachings of the reference relative to *in vitro* inhibition of EMCV to *in vivo* treatment of HCV in subjects.

Accordingly, even though the reference asserts an ability to treat HCV using a polynucleotide vector encoding omega interferon, the reference does not provide an enabling disclosure in this regard. I am not aware of any U.S. Food and Drug Administration (FDA) approved polynucleotide expression based treatment methods where the polynucleotide

encodes alpha interferon. Further, I am not aware of any on-going clinical trials with such treatment. Treatment of chronic HCV infection with pegylated alpha interferon protein plus ribavirin is the current standard of care.

10. Further, protein expression levels from polynucleotide constructs used for gene therapy are notoriously unreliable. Even in the year 2005 (about six years after the filing date of the Parker, et al., reference), meeting notes from the American Society of Gene Therapy, "Challenges in Advancing the Field of Gene Therapy: A Critical Review of the Science, Medicine, and Regulation" (Appendix B) discussed the many barriers to effective gene therapy. For example, Katherine High (Children's Hospital of Philadelphia) noted that "secondary scientific problems have emerged, such as tissue-specific immune responses elicited against the vector and transgene and the need for targeted delivery to specific organs or tissues" (*see* page 1). Successful use of gene vectors for delivery of therapeutic agents in humans has been troublesome and complicated (*see* pages 2-3). Further, there have been very few gene therapy successes. As noted by Alan Kinniburgh (National Hemophilia Foundation), there are "relatively few gene therapy successes that have been achieved" (*see* page 10). The meeting notes point out numerous, unaddressed difficulties relating to innate immunity and the paradoxes of viral pathogens and tissue injury in gene therapy (*see* pages 5-6). Safety is also a large concern and unanswered problem of gene therapy (*see* comments of Daniel Rosenblum (OCTGT/FDA), pages 14-15). Daniel Salomon (The Scripps Research Institute) noted the "persisting uncertainty regarding the selection of appropriate animal models requires resolution and more rigorous pharmacokinetic and pharmacodynamic analyses of gene transfer systems are needed" (*see* page 17). Conclusions from this meeting were generally that gene therapy is in very early stages and that "[i]dentification and mechanistic understandings of the problems the field now faces in vectorology, insertional mutagenesis, and immunity are necessary first steps to designing strategies to successfully address these challenges" (*see* page 18; emphasis added).

Accordingly, the Examiner's statement that "the polynucleotide of Parker would be expected to be expressed and translated into a therapeutically effective amount of IFN-ω, and this dose of IFN-ω would be expected to fall within the claimed dose ranges" (*see* Office action, mailed 28 May 2008, page 5) is completely unsubstantiated by the reference of

Parker, et al. The Examiner has not provided any prior art supported evidence teaching predictable, sustained levels of protein expression in an animal subject using polynucleotide constructs. In fact, the Examiner's assertion of predictability appears to be contrary to the general state of the art of gene therapy as late as 2005, which is about six years after the publication of the reference of Parker, et al.

11. Second, I am familiar with the teachings of the reference of Goeddel, et al. In the Office action, mailed 28 May 2008, the Examiner asserts that the reference "teaches IFN-ω proteins with biological activities, such as antiviral activity, that are similar to the IFN-α that is also used to treat HCV" (*see, e.g.,* Office action, mailed 28 May 2008, page 4). The reference characterizes the *in vitro* antiviral activity of omega interferon compared to alpha interferon against Vesicular Stomatitis Virus (VSV) (a *Rhabdoviridae* virus) and Encephalomyocarditis Virus (a *Picornaviridae* virus) (*see* cols. 7-8 and 10) -- not against HCV (a *Flaviviridae* virus). No *in vivo* data is presented. Extrapolating the teachings of the reference to assert that, because one interferon is an effective antiviral against a particular virus, the same will be true for other interferons is not reasonable and is not substantiated by what is known about various interferons. The Examiner is making the assumption that interferons are interchangeable in their pharmaceutical activities. This assertion is unsupported by the Examiner and is inconsistent with what is known regarding omega interferon.

12. Omega interferon and alpha interferon have very different physical and *in vitro* properties, for example: the sequences have only 62% sequence identity -- extrapolation of pharmaceutical activity based on this level of sequence homology is not reasonable. Alpha interferon and omega interferon have different solubilities and different stabilities in aqueous solutions. Omega interferon has been shown to be less effective in anti-cell proliferation assays relative to alpha interferon. Based on their physical and *in vitro* properties alone there no reason for a typical scientist to expect that they would perform the same in therapeutic applications.

13. As noted by Viscomi (in "Structure-activity of type I Interferons," Biotherapy 10:59-86 (1997); Appendix C) omega interferon and alpha interferon can show distinct properties and significant variety in their biological actions:

> the members of that family, IFNα species, IFNβ and IFNω, due to local differences in the structure sometime show distinct properties. From the reported data it results that even minute changes or differences in the primary sequences could be responsible for a significant variety of biological actions, thus inducing to the hypothesis that Type I IFNs, rather than to be the result of a redundant replication during the evolution, play definite roles in the defense of living organisms to foreign agents. (*See* Abstract; emphasis added.)

Further, the reference of Buckwold, et al., of which I am a co-author (Antiviral Res. 2007 Feb;73(2):118-25.; Appendix D; already of record in the present application), points out several distinct differences between the antiviral activities of alpha, beta, gamma, and omega interferons, for example: omega interferon was more active than alpha interferon against Bovine Viral Diarrhea Virus (BVDV) and beta and gamma interferon were not effective against BVDV (*see* page 120, col. 1). Glycosylated omega interferon was slightly more potent than gamma interferon and more potent than alpha interferon against Yellow Fever Virus (*see* page 120, cols. 1-2). Regarding West Nile Virus, omega interferon was also more potent than alpha interferon (*see* page 120, col. 2).

Accordingly, the cited prior art contradicts the Examiner's assertion that a typical scientist would expect that, because one type of interferon demonstrated a particular therapeutic effect, all other interferons in the same class would necessarily have the same effect.

14. Yet another argument against omega interferon and alpha interferon having equivalent pharmaceutical activity is found in the teachings of the present specification, which discusses successful treatment of human patients with omega interferon who failed treatment using alpha interferon (*see, e.g.,* Specification page 17, ¶¶0058-0059). Even if, for the sake of argument, there would be some expectation of equivalence of function between alpha interferon and omega interferon, this result demonstrates that there is no predictable expectation of equivalence of function when alpha interferon is used in the treatment of HCV versus when omega interferon is used.

15. Intarcia Therapeutics, Inc., has performed clinical trials in humans demonstrating the activity and tolerability of administration of omega interferon protein by injection and use of subcutaneous implants for the treatment of HCV. As noted above, the

present specification discusses successful treatment of human patients who failed treatment using alpha interferon, as well as those who were treatment naive (*see, e.g.*, Specification page 17, ¶¶0058-0059). Clinical trial data for treatment of HCV with injected omega interferon protein is also shown in the poster presented at the Digestive Disease Week, May 21 2007 Meeting (Appendix E). The data demonstrated that treatment with omega interferon protein was well-tolerated by patients and demonstrated excellent anti-HCV activity. The poster also illustrates an ongoing clinical trial, sponsored by Intarcia Therapeutics, Inc., using an implantable osmotic delivery system (DUROS®; ALZA Corporation, Mountain View, CA) to deliver omega interferon protein continuously over extended time periods to patients who have failed the standard treatment of pegylated alpha interferon plus ribavirin. Current results from this clinical trial also demonstrate that treatment of these patients (i.e., patients whose HCV infection exhibit primary or secondary resistance to treatment with alpha interferon) with omega interferon protein is well-tolerated by patients and provides excellent anti-HCV activity.

16. As noted above in the discussion of my employment history, I worked on therapeutic applications of INTRON® A (interferon alfa-2b), including treatment of HCV infection using interferon alfa-2b. Interferon alfa-2b was approved for treatment of HCV infection in 1991. The reference of Goeddel, et al., claims priority back to 1984, with related publications by the Japanese and European Patent Offices in 1986. Further, the sequence of omega interferon was published by Hauptmann, et al., in 1985 (Nucleic Acids Res. 13:4739-4749 (1985)). Thus omega interferon was known to the typical scientist as of the mid-1980s. I am not aware of any discussion in the scientific literature or published use of omega interferon protein as a treatment for HCV infection, prior to the work performed at Intarcia Therapeutics, Inc. (formerly BioMedicines, Inc.).

17. In my opinion, in view of my remarks herein above and in the absence of any prior *in vitro* data and in particular in the absence of clinical data on the usefulness of the administration of omega interferon protein for the treatment of HCV, any assertion of the probable efficacy of such treatment based on the cited references is no more than wishful thinking. These references do not support a finding that a typical scientist at the time the invention was made would have assumed that administration of omega interferon protein

would be efficacious for the treatment of HCV based on the assertion of the Parker, et al., reference that a polynucleotide encoding omega interferon might be used to treat HCV. Further, the references cited by the Examiner do not support a finding that a typical scientist at the time the invention was made would assume that omega interferon protein would be expected to perform all the same functions as alpha interferon. These references do not support a finding that a typical scientist at the time the invention was made would be "motivated" to administer omega interferon protein for the treatment of HCV rather than the standard of treatment of alpha interferon protein. Finally, these references do not support a finding that a typical scientist at the time the invention was made would have recognized that the HCV treatment results obtained by the claimed methods were predictable.

18. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

23 October 2008
Date

Thomas R. Alessi, Ph.D.

Appendix A
Curriculum Vitae

THOMAS R. ALESSI, PH.D.

Intarcia Therapeutics, Inc.
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Hayward, California 94541
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Education

Ph.D. Organic Chemistry	University of Rochester	1982 - 1986
M.S. Organic Chemistry	University of Rochester	1980 - 1982
B.S. Chemistry	Allegheny College	1976 - 1980

Honors

Phi Beta Kappa, 1979
Sherman Clarke Fellow, 1980-1985
Allegheny Merit Scholarship, 1976-1980
Magna Cum Laude, Allegheny College, 1980
American Institute of Chemists Award, 1980
ACS Analytical Chemistry Award, 1979

Intarcia Therapeutics, Inc., Emeryville, California

Vice President, Development and Manufacturing	August 2007 – present
Vice President, Development	February 2002 – August 2007
Executive Director, Regulatory Affairs and Product Development	December 1997 - January 2002

Responsibilities:

- Senior management of groups responsible for GMP manufacturing of clinical drug supplies, product development, and regulatory affairs
- partner management for company drug development programs with ALZA, Boehringer-Ingelheim, and Schering AG
- vendor management of manufacturing sites, analytical laboratories, drug labeling, packaging, and distribution vendors, and preclinical vendors
- oversight of GMP manufacturing of bulk drug substances and clinical drug supplies at contract manufacturers
- program management through organization and direction of formal drug development teams

Responsibilities: (continued)

- negotiation of contracts with vendors and investigators
- presentations to the board of directors, partners, and potential investors
- due diligence for in-licensing potential drug products
- review, approval, and development of preclinical protocols and reports
- liaison to FDA CDER Oncology Products Division & Antiviral Drugs
- direct, review, approval, and preparation of regulatory documents

Ansan Pharmaceuticals, Inc., South San Francisco, California

Senior Director, Regulatory Affairs, Quality Assurance, & Project Management — August 1995 - November 1997

Responsibilities:

- regulatory strategy
- liaison to FDA CDER Oncology, Gastrointestinal and Coagulation, and Dermal and Dental Drug Products Divisions
- review, approval, and preparation of regulatory documents
- review, approval, and development of clinical protocols, investigator's brochures, and case report forms
- compliance with current good manufacturing practices (cGMPs)
- quality assurance (QA) oversight of contract manufacturing
- review, approval and development of chemistry, manufacturing, and controls (CMC) documents
- management of five drug development programs and budgets
- negotiation of agreements with third-party suppliers
- review, approval, and development of preclinical protocols
- presentations and correspondence to the board of directors and potential corporate partners
- due diligence for in-licensing potential drug products

Glycomed Incorporated, Alameda, California

Director, Regulatory Affairs and Quality Assurance	October 1994 - June 1995
Associate Director, Quality Assurance	April 1994 - October 1994
Associate Director, Regulatory Affairs	September 1993 - April 1994

Responsibilities:
- regulatory strategy and liaison to FDA
- review, approval, and directing preparation of regulatory documents
- regulatory and QA guidance to research and development
- QA interface with contract manufacturers and laboratories
- compliance with cGMPs

Schering-Plough Research Institute, Kenilworth, New Jersey

Senior Manager, U.S. Regulatory Affairs	April 1992 - August 1993
Manager, U.S. Regulatory Affairs	May 1990 - April 1992

Responsibilities:
- regulatory strategy for chemistry and manufacturing issues
- liaison for INTRON A (interferon alfa-2b) to CBER Biological Response Modifiers Group for hepatitis C and hepatitis B programs
- liaison to FDA CDER Pulmonary, Pilot Drug, and Anti-Infective Drug Products Divisions and Office of Generic Drugs
- review, approval, and directing preparation of regulatory documents
- regulatory guidance to research, development, and manufacturing
- review and approval of clinical study protocols and study reports
- review and approval of drug labeling, advertising, and promotion
- training of regulatory affairs managers and assistants

Wyeth-Ayerst Research, Princeton, New Jersey

Research Scientist, Medicinal Chemistry	July 1988 - May 1990
Senior Scientist, Medicinal Chemistry	March 1985 - July 1988

Responsibilities:
- rational design of insulin sensitivity enhancers and aldose reductase inhibitors
- synthesis of novel biologically active molecules
- chemistry representative to antihyperglycemic project team
- screening, recruiting, and training of B.S./M.S. research chemists

ACCOMPLISHMENTS

Development and Program Management

- Directed joint Intarcia-ALZA partnership team for development of Omega DUROS
- Successfully re-established GMP manufacturing of clinical drug supplies for three products in-licensed by Intarcia in support of new Phase 1b, Phase 2, and Phase 3 clinical trials. Successfully directed technology transfer of manufacturing and analytical methods to new vendors.
- Directed improved synthesis of bulk drug substance and successful switch to more stable polymorphic form of Biomed 101.
- Designed and directed pharmacology studies that established proof-of-concept for new indication for Biomed 101 and supported filing of new and subsequently issued patent.
- Established formal multidiscipline product development teams at Intarcia.
- Coordinated drug development activities to bring three drug programs from preclinical to IND stage of development within a 12-month period at Ansan.
- Developed, reviewed, and approved protocols for pharmacology, toxicology, and genotoxicity studies needed to support IND filings. Negotiated agreements with vendors to conduct the studies. Reviewed, edited, and approved final study reports.
- Managed preparation and review of study reports for the pivotal clinical trials to support approval of INTRON A for two new indications. Saved the company three to six months by beating FDA deadlines for Advisory Committee meeting.
- Coordinated efforts of technical and medical reviewers, proof-readers, documentation group, and contract printers to achieve overnight turnaround of draft and final labeling to support approval of INTRON A for two new indications.
- Reviewed and coordinated preparation of all labeling and supporting documentation for FDA approval of five new INTRON A packages.

Regulatory Affairs

- Negotiated FDA approval of INTRON A (Interferon alfa-2b, recombinant) for *two new* indications; Chronic Hepatitis C (Feb 1991) and Chronic Hepatitis B (July 1992).
- Coordinated presentations for two successful FDA advisory committee meetings; each resulting in a unanimous recommendation for approval.
- Negotiated approval for launch of new Vancenase Pockethaler Nasal Inhaler prior to FDA review of NDA supplement, saving the company approximately six months worth of sales.
- Reviewed, edited, and co-authored five new INDs; reviewed and critiqued three other new INDs and two new NDAs.

- Successfully negotiated with FDA to transfer and update INDs for two drugs in-licensed by Intarcia to new reviewing divisions for new indications. Saved cost and timing to create new INDs for these products.

- While at Ansan, held three successful pre-IND meetings with three different divisions of FDA within six months. Reviewed, edited, and co-authored briefing documents for FDA.

- Successfully obtained FDA feedback on issues concerning an investigational new drug without a pre-IND meeting, saving Glycomed one to three months. Coordinated preparation of what FDA called "one of the best pre-IND documents we've ever seen!"

- Reviewed and approved all labeling, advertising, and promotional materials in support of approval of INTRON A for two new indications.

- Provided regulatory support for approximately 25 active INDs and 65 approved NDA drug products. Responsible for review and assessment of regulatory impact of proposed manufacturing changes for all Schering products.

Quality Assurance

- Schering representative during FDA clinical sponsor audit that resulted in no "483" issued.

- Established Quality Assurance function at Intarcia, Glycomed, and Ansan.

- Assessment of technical capabilities and GMP compliance of a new manufacturer enabled selection of that vendor by project team and resulted in a savings of about 6 months and $100,000.

- Facilitated preparation of three GMP "registration" batches by working closely with drug substance manufacturer to develop and review batch records and analytical procedures.

- Upon joining Ansan, disqualified a drug supplier based on a GMP audit and rejected existing clinical supplies. Successfully qualified a new supplier and coordinated the manufacturing, release and stability testing of new drug product for start of the trial.

Other Accomplishments

- Successfully designed and rewrote new "user-friendly" Package Insert (PI) for INTRON A that was approved by FDA. The PI, containing five different indications, was approved by five company project teams and multiple FDA reviewers and was praised by clinicians.
- Initiated project to review and collate CMC sections of NDAs for 65 approved products.
- Successfully lead efforts to evaluate and standardize software used by the company.
- Created database to track specifications and procedures and to provide clear audit trail for documentation.
- Created easy-to-use templates that control the format and style of regulatory, clinical, and preclinical documents. Templates facilitate preparation of the documents and standardize the content.
- Created flexible "user-friendly" database to collect, organize, and present information on drug product formulations and stability. Database enabled identification of possible solutions to formulation problems.
- Established and managed local area computer networks at Wyeth-Ayerst and at Ansan.

Rational Drug Design

- Discovered series of novel antihyperglycemic agents. Through structural modifications, prepared compound that was *100 times more active* than initial lead within eight months. New compound was selected for IND development.

Patents

Moran, S.M.; Alessi, T.R. "Methods of Using Butyric Acid Derivatives to Protect Against Hair Loss," Patent Number 5,962,523, October 5, 1999.

Alessi, T.R.; Dolak, T.M.; Ellingboe, J.W.; Lombardo, L.J. "Novel Naphthalenylalkyl-3H-1,2,3,5-oxathiadiazole 2-oxides Useful as Antihyperglycemic Agents," Patent Number 4,897,405, January 30, 1990.

Alessi, T.R.; Dolak, T.M. "Novel Benzyl-3H-1,2,3,5-oxathiadiazole 2-oxides Useful as Antihyperglycemic Agents," Patent Number 4,895,862, January 23, 1990.

Alessi, T.R.; Ellingboe, J.W. "Novel Substituted-3H-1,2,3,5-oxathiadiazole 2-oxides Useful as Antihyperglycemic Agents," Patent Number 4,895,860, January 23, 1990.

Lombardo, L.J.; Alessi, T.R. "Novel Naphthalenyl-3H-1,2,3,5-oxathiadiazole 2-oxides Useful as Antihyperglycemic Agents," Patent Number 4,895,861, January 23, 1990.

Ellingboe, J.W.; Bagli, J., Alessi, T.R. "Process for the Preparation of Novel Naphthylenylmethyl-3H-1,2,3,5-oxathiadiazole 2-oxides Useful as Antihyperglycemic Agents," Patent Number 4,966,975, October 30, 1990.

Bagli, J.; Ellingboe, J.W., Alessi, T.R. "Preparation of 2,4-Disubstituted-5-cyano-4-pyrimidinyloxyacetic acid Aldose Reductase Inhibitors," Patent Number 4,906,753, March 6, 1990.

Bagli, J.; Ellingboe, J.W., Alessi, T.R. "2,4-Disubstituted-5-cyano-1,6-dihydro-6-oxo-1-pyrimidineacetic acid Aldose Reductase Inhibitors," Patent Number 4,900,829, February 13, 1990.

Bagli, J.; Ellingboe, J.W., Alessi, T.R. "2,4-Disubstituted-5-cyano-1,6-dihydro-6-oxo-1-pyrimidineacetic acid Aldose Reductase Inhibitors," Patent Number 4,786,638, November 22, 1988.

Bagli, J.; Ellingboe, J.W.; Alessi, T.R. "2,6-Disubstituted-5-cyano-4-pyrimidinyloxyacetic acid Aldose Reductase Inhibitors," Patent Number 4,786,640, November 22, 1988.

Publications and Abstracts

Buckwold, V.E., Wei, J., Huang, Z., Huang, C., Nalca, A., Wells, J., Russell, J. Collins, B., Ptak, R., Lang, W., Scribner, C., Blanchett, D., Alessi, T., Langecker, P. "Antiviral activity of CHO-SS cell-derived human omega interferon and other human interferons against HCV RNA replicons and related viruses." *Antiviral Res.* **2007**, *73*, 118.

Buckwold, V.E., Wei, J., Huang, Z., Huang, C., Nalca, A., Wells, J., Russell, J., Lang, W., Scribner, C., Blanchett, D., Alessi, T., Langecker, P. "Antiviral activity of CHO-SS-derived omega interferon and other human interferons against HCV RNA replicons and related viruses." *Abstract #652* presentation at DDW, May 21, **2006**.

Rohloff, C.M., Alessi, T.R., Yang, B., Dahms, J., Carr, J.P., Scott D. Lautenbach, S.D. "DUROS® Technology Delivers Peptides and Proteins at Consistent Rate Continuously for 3 to 12 Months." *J. Diabetes Sci.Technol.* **2008**; *2(3),* 461.

Yang, B., Rohloff, C., Mercer, R., Horwege, K., Negulescu, C., Lautenbach, S., Gumucio, J., Gou, M., Weeks, E., Carr, J., Ford, D., Alessi, T. "Continuous Delivery of Stabilized Proteins and Peptides at Consistent Rates for at least 3 months from the DUROS® Device." *Abstract AM-08-01934* accepted for poster presentation at AAPS, November 16, **2008**.

Novozhenov, V., Zakharova, N., Vinogradova, E., Nikitin, I., Gorbakov, V., Yakovlev, A., Pak, S., Rafalski, V., Bogomolov, P., Alessi, T., Blanchett, D., Lang, W., Langecker, P., McNally, J., Bacon, B. "Phase 2 Study of Omega Interferon Alone or in Combination with Ribavirin in Subjects with Chronic Hepatitis C Genotype-1 Infection." *Abstract M1798* poster presentation at DDW, May 21, **2007**.

Ellingboe, J.W.; Alessi, T.R.; Dolak, T.M.; Nguyen, T.T.; Tomer, J.D.; Guzzo, F.; Bagli, J.F.; McCaleb, M.L. "Antihyperglycemic Activity of Novel Substituted 3H-1,2,3,5-Oxathiadiazole 2-Oxides." *J. Med. Chem.* **1992**, *35,* 1176.

Ellingboe, J.; Alessi, T.R.; Millen, J.; Sredy, J.; King, A.; Prusiewicz, C.; Guzzo, F.; VanEngen, D.; Bagli, J. *J. Med Chem.*, **1990**, *33,* 2892.

Boeckman, R.K. Jr.; Springer, D.M.; Alessi, T.R. "Synthetic Studies Directed Toward Naturally Occurring Cyclooctanoids. 2. A Stereocontrolled Assembly of (±)-Pleuromutilin *via* a Remarkable Sterically Demanding Oxy-Cope Rearrangement." *J. Am. Chem. Soc.*, **1989**, *111,* 8286.

Boeckman, R.K. Jr.; Alessi, T.R. "Stereocontrol in the Intramolecular Diels-Alder Reaction. 3. A Potentially General Method for the Synthesis of *cis*-Hydrindenes by Use of (Z)-Diene Units." *J. Am. Chem. Soc.*, **1982**, *104,* 3216.

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Appendix B

American Society of Gene Therapy, "Challenges in Advancing the Field of Gene Therapy: A Critical Review of the Science, Medicine, and Regulation"

AMERICAN
SOCIETY of
GENE
THERAPY

Challenges in Advancing the Field of Gene Therapy: A Critical Review of the Science, Medicine, and Regulation

Arlington, VA, USA, April 7-8, 2005

Kara A. Nyberg, Katherine A. High, and Daniel R. Salomon

The 225 participants attending the 2005 American Society of Gene Therapy (ASGT) Stakeholders' Meeting held in Washington, D.C., on April 7 and 8 were greeted by warm weather, a city in full cherry-blossom bloom, and an enthusiastic organizing committee. The focus of this year's meeting centered on scientific, medical, and regulatory challenges in advancing the field of gene therapy, which drew members from academia, industry, government agencies, disease foundations, and the press. The ASGT jointly sponsored the meeting with the Center for Cellular and Molecular Therapeutics at the Children's Hospital of Philadelphia, with additional support from Genzyme, the Biologics Consulting Group, and Avigen. This article summarizes clinical gene transfer barriers faced by those in the field and possible strategies for circumvention as discussed in a series of presentations focusing on vectorology, immunology, vaccine design, and real stories.

Introduction

Katherine High (Children's Hospital of Philadelphia), co-chair of the conference along with Daniel Salomon (The Scripps Research Institute), began by emphasizing that the goal of the conference was to identify and discuss the challenges to advancing clinical gene transfer research. In sum, the field is currently progressing only slowly despite a significant medical need and is beset by a general but false perception that gene therapy has not been successful. She delineated several obstacles to progress that would be revisited many times throughout the 2-day forum.

Although the principles behind gene transfer are sound, High noted that secondary scientific problems have emerged, such as tissue-specific immune responses elicited against the vector and transgene and the need for targeted delivery to specific organs or tissues. The changing climate for clinical investigation in U.S. multi-center trials also presents a major obstacle. For example, the multi-layer review process slows approvals, and institutional review boards (IRBs) from participating institutions, with individualized rules and regulations, further complicate and delay the review process. Notably, a small number of unexpected serious adverse events have caused increased federal and IRB scrutiny of trials, which further slows timelines, sucks up funding, contributes to an increased sense of risk, and forces many smaller biotech companies to drop out of the process entirely. Financial hurdles also present obstacles, since big pharmaceutical companies typically do not enter the clinical trials process and provide funding until phase 3 trials are underway. This leaves principal investigators (PIs), small biotech companies, and disease foundations to foot the bill for pre-clinical, phase 1, and phase 2 studies.

As early-phase clinical trials become more frequently based at academic medical centers, adjustments in staffing will be needed since clinical trial research is not easily divided into projects for post-doctoral researchers and fellows. In addition, the responsibility for pushing the clinical trial timeline forward falls solely on the shoulders of the PI backing the trial—an unsatisfactory arrangement since these individuals typically bear many other responsibilities and are often not prepared for or experienced with the rigorous formal conduct required for enabling a phase 2 clinical trial. At a minimum, this situation constitutes a serious disincentive for the participation of new investigators who will represent the next generation of gene therapy researchers.

Trial momentum can degenerate into a jerky ride of starts and stops in the face of recurrent clinical holds. High noted, however, that as experiments with a given class of vectors proceed and knowledge is accrued, clinical studies are expected to progress more smoothly. Commercial obstacles include complications arising from intellectual property issues that limit information sharing prior to commercial development and industry pressure to develop therapies for large-market indications, even though these may not be the most straightforward choices from a scientific standpoint. Other hurdles High mentioned include the scalability, manufacture, and access to clinical-grade vector and public perceptions of the relative risks involved in participating in gene transfer studies.

In conclusion, High compared the current problems confronted in advancing clinical gene transfer trials to those tackled not long ago by researchers studying monoclonal antibodies as a class of therapeutics. The therapeutic potential of this technology was appreciated, but years of clinical trial failure prevented its clinical application. Indeed, the considered wisdom at one point was that there was little or no potential in this area, and many large pharmaceutical companies walked away from millions of dollars in investments and intellectual property. Slowly but surely, however, problems were identified and solved, thereby eventually permitting the huge and growing clinical and commercial success today. It appears that the field of therapeutic gene transfer is at a similar crossroads today.

Vectorology

Arthur Nienhuis (St. Jude Children's Research Hospital) and Barrie Carter (Targeted Genetics Corporation) co-chaired the first session on vectorology barriers to gene transfer clinical trials, which included six presentations covering the range of gene transfer vectors currently used in experimental studies. David Williams (Cincinnati Children's Hospital Medical Center) began with a discussion of retroviral and lentiviral vectors employed in gene transfer studies of Fanconi's anemia (FA), a disease typically affecting children characterized by progressive bone marrow failure due to mutations in any of 11 different genes. Roughly 30% of patients with a human leukocyte antigen (HLA) matched sibling donor receive the standard FA therapy of bone marrow transplantation. For the remaining 70%, the next-best therapy—stem cell transplantation using unrelated donors—has a high associated morbidity and mortality. Consequently, corrective gene transfer using a retrovirus vector presents an attractive, alternative therapeutic option, especially given the efficacy demonstrated in the FA mouse model. Carrying this success over to humans, however, has been troublesome, as ex vivo manipulation of FA hematopoietic stem cells (HSC) is complicated by their genetic instability and low abundance at the time of critical disease progression. To ameliorate these difficulties, Williams says that he and his colleagues have been able to obtain a better source of HSC by collecting the cells before the onset of aplastic anemia, isolating more cells during collection, and optimizing cryopreservation and thawing procedures. In vitro cell culture has also been improved by

implementing shorter culture times and better cytokines, while gene transfer efficiency has been greatly enhanced by using a high-titer murine leukemia virus vector pseudotype with a Gibbon ape leukemia virus (GALV) envelope protein, which is administered in one to two exposures over 36 hours. Several FA genotype A children with no HLA-identical sibling donors are currently enrolled in a pilot trial employing a murine stem cell virus backbone. This study encountered no significant regulatory obstacles prior to trial initiation, although the investigators had to extensively evaluate patients for clonal hematopoiesis following the serious adverse events seen in the French X-linked severe combined immunodeficiency (X-SCID) trial.

In light of the adverse events of the X-SCID trial, Cynthia Dunbar (National Heart, Lung, and Blood Institute, NIH) presented data from long-term non-human primate studies regarding whether integration toxicity in humans is cause for concern. She began by throwing out a question many wonder about: Is gene therapy with integrating vectors safe? Forty-six rhesus macaques receiving stem cells transduced with retroviral vectors have been studied for a median follow-up of 4.5 years—representative of 6,000 "insertion years"—and during this time no clinical or molecular evidence for progression to a pre-leukemic or a leukemic state has yet been observed. However, one 3-year-old macaque that received a retrovirally transduced stem cell transplant following total body irradiation in 1999 demonstrated an adverse event 5 years later. In 2000, this animal exhibited an unusual gene-marking pattern, with two dominant clones identified—one in chromosome 15 and the other in chromosome 19. In 2001, the animal received 1 dose of cytotoxic drug treatment. The animal demonstrated normal blood counts every 6 months until September 2004 when the animal's health rapidly deteriorated and it died. Necropsy analysis revealed myeloid sarcoma infiltration, and molecular evidence for elevated vector levels in the tumor was found. The retroviral vector inserted at the locus encoding BFL-1, an anti-apoptotic member of the BCL2 family that prolongs cell survival while allowing for proliferation and some differentiation. Hence, retrovirus integration at this locus possibly induced over-expression of BFL-1 in the kidney tissue, thereby precipitating tumor formation. Subsequent mapping of 702 insertions in the other treated monkeys revealed a significant over-representation (1.8%) of independent insertions at the MDS1-EVI1 locus in 9 different animals. The MDS1 (MyeloDysplasia Syndrome 1) and EVI1 (Ectopic Virus Integration 1) genes encode transcription factors implicated in human leukemias. Despite the propensity to integrate at this locus, no evidence of clonal expansion in 5 individual clones has yet been observed for periods up to 7 years. Dunbar concluded that these integration events do not represent integration at a "hot-spot." She also believes that lifelong follow-up is required for those treated with integrating, persisting vectors, and this analysis should be complemented by prolonged non-human primate and murine studies.

Philip Gregory (Sangamo BioSciences) next turned the discussion toward gene correction as an alternative therapeutic modality. Instead of gene addition, gene correction provides site-specific, permanent modification of the genome using transient delivery of the corrective vector system and requires no need for ectopic gene insertion or a heterologous promoter. Zinc finger DNA binding protein nucleases (ZFNs) are genome editors that can be harnessed to promote gene correction, gene disruption, and site-specific integration. For example, designer nucleases combining zinc finger proteins and Fok1 (a non-specific restriction endonuclease) have been created in which the ZFNs bind to a targeted DNA sequence and cleave the DNA to cause a double-strand break. Homologous recombination in human cells occurs in approximately 1×10^6 cells; however, homology-directed repair of a double-strand break using a sister chromatid as a template is more than 1000-fold more efficient. The ZFN system can not only create a double-strand break at a site-specific location to induce the process of homology-directed repair, but donor plasmid DNA can also be transferred into the cells to further control the gene correction

process in the event that the intact sister chromatid is defective. Initial ZFN studies in cell culture and human T cells have provided encouraging results when correcting IL2R (Interleukin-2 Receptor) gene mutations related to X-SCID. But what obstacles might hinder clinical applications of this technology? First, Gregory explained that designing potent ZFNs is non-trivial when factoring in the specificity and architecture of the system. Second, the range of mutations or deletions that can be targeted is restricted since the efficiency of conversion becomes greatly diminished when the mutation and ZFN cleavage site are greater than 100 base pairs apart. Third, the current delivery approach is ex vivo, making either plasmid DNA or viral systems the best methods for ZFN delivery—both of which require additional research. Moreover, researchers have yet to demonstrate system safety and efficacy in appropriate model organisms.

In turning to adeno-associated virus (AAV) vectors, Terence Flotte (University of Florida) stated that continuous identification and management of long-term gene transfer risks associated with recombinant AAV vectors is needed, specifically focusing on insertional mutagenesis, inadvertent germ-line transmission, and immune responses to capsids and transgene products. AAV vectors are typically used for long-duration gene transfer, and more than 110 serotypes have been identified in humans and non-human primates since the virus lives symbiotically within primates. Flotte's research is specifically focused on constructing a safe and effective AAV vector system to treat patients deficient for Alpha-1 Antitrypsin (AAT), a cause of genetic emphysema. Initial studies of murine skeletal muscle transduced with AAT using an AAV2 vector demonstrated long-term, functional AAT protein secretion from muscle cells. These analyses progressed to a phase 1 study beginning in March 2004 focused on (a) assessing the safety of intramuscular administration of human AAT in adult AAT-deficient patients and (b) determining the dose of the vector-transgene system required to achieve a detectable level of AAT in these patients. Based on preliminary findings, 8 patients have been safely treated, and no serious vector-related adverse events have been observed. Serum muscular AAT levels are initially elevated but then diminish over time, anti-AAV2 antibodies develop steadily over time, and no anti-AAT antibodies have been detected thus far. These subjects are scheduled to participate in long-term follow-up for 15 years, so future data will provide more insight. The only obstacle Flotte mentioned in moving the research to the clinic was compliance with an extensive good manufacturing practice (GMP) scheme for the AAV2 vector, although this is now being refined.

Arthur Beaudet (Baylor College of Medicine) presented a discussion of helper-dependent adenoviral (HDAd) vectors for liver- and lung-directed gene therapy. Similar to Flotte, Beaudet has devoted much time and effort to improving the quantity and quality of HDAd vector production. The major obstacle with translating adenoviral-based vectors to the clinic, however, has been substantial vector toxicity. Initial adenoviral toxicity studies revealed acute toxicity within the first 24 hours after vector injection, followed by viral gene expression from Days 2-4. An immune response then arose during Weeks 2-6 when chronic viral expression was in effect. In humans and non-human primate studies, the efficiency of adenovirus-mediated hepatic transduction following systemic injection increased with increasing vector doses, but so too did toxicity. Subsequent toxicity-limiting studies in mice revealed that hydrodynamic injection of HDAd resulted in increased hepatic transduction, reduced systemic vector dissemination, and less severe elevation of pro-inflammatory cytokines than conventional injection. Hydrodynamic injection into baboons using a balloon system to block hepatic venous outflow to thereby increase intrahepatic pressure also demonstrated stable, long-term, high-level transgene expression with no chronic toxicity. Studies of HDAd-mediated, lung-directed gene therapy are also being carried out in baboons using a pressure-actuated Intratracheal AeroProbe aerosol

delivery catheter. Thus far, the vector doses administered have been well tolerated with minimal toxicity, and widespread transgene expression is seen. Based on these data, Beaudet hopes to initiate a clinical trial for hemophilia A or B and a clinical trial for cystic fibrosis by February 2007. Before these trials can proceed, however, the Baylor group must achieve clinical grade vector production in addition to gaining regulatory approval.

Joseph Glorioso (University of Pittsburgh School of Medicine) ended the vectorology section with a presentation on replication-defective herpes simplex virus (RD-HSV) vectors and their use in treating pain and neuropathy associated with sensory nerve disease. Use of RD-HSV vectors in neuronal tissues is advantageous given the large load capacity of the vector, targeted delivery to sensory ganglia by retrograde transport, stable persistence in neurons in a non-integrated state, a lack of neural toxicity, short- or long-term gene expression, and scalable vector manufacture with no wild-type recombinants produced. Glorioso and colleagues are employing the HSV vector to treat chronic pain, an unmet medical need that affects 60 million Americans. Nociceptors transmit noxious stimuli, which are ultimately transmitted to the spinal cord and brain. To block nociceptor signaling, mice were transduced with HSV-mediated proenkephalin (vector SHPE), which indeed reduced mechanical allodynia (pain caused by a normally non-painful stimulus) in a spinal nerve ligation model of neuropathic pain. Moreover, gene transfer did not induce tolerance and was additive with morphine treatment. These preclinical results support a phase 1/2 randomized dose-escalation study in humans to evaluate the safety and clinical efficacy of the vector SHPE system. Other uses of RD-HSV technology include reversing diabetic neuropathy via axon regeneration using HSV-mediated delivery of nerve growth factor or neurotrophin-3. One of the major rate-limiting steps for clinical trial assessment of this technology is HSV vector manufacture.

Immunology of Gene Therapy

The next session topic, co-chaired by Savio Woo (Mount Sanai School of Medicine) and Michel Sadelain (Memorial Sloan-Kettering Cancer Center), focused on the problems associated with gene therapy immunology. Daniel Salomon, a conference co-chair, began with a frank discussion of innate immunity and the paradoxes of viral pathogens and tissue injury in gene therapy. He reminded the audience that (a) many gene delivery vectors are built on pathogen skeletons, some of which still retain pathogenic genes; (b) the delivery of therapeutic genes, whether by injection/inhalation or ex vivo cell separation and manipulation, often causes direct tissue injury; and (c) therapeutic gene delivery by nature will often alter the state of the target cell and/or host. These combined factors often induce cells to produce "danger signals." Innate immunity is the body's first line of defense to acute injury and occurs within the first 12 hours of invasion; it is primitive, operates by pattern-recognition receptors, and has no memory. In contrast, adaptive immunity, activated next, represents a complex system that responds to specific antigens; it evolves, operates by determining self from non-self, and has memory. Innate immunity and adaptive immunity are linked such that activation of innate immunity will induce activation of adaptive immunity. Salomon stressed that innate immunity is good in the context of therapeutic vaccines, as it leads to enhanced adaptive immunity, both locally and systemically. In the context of a tumor vaccine or therapy, innate immunity could also trigger a very acute, tumor-destructive local response. In addition, bestowing a competitive advantage on gene-modified cells could enhance the selection of healthy over diseased cells. In contrast, he argued that innate immunity could be bad if tissue injury created at a transplant site triggers these potent natural defense mechanisms and targets the host cells expressing viral proteins for destruction by the immune system. To help move the gene therapy field forward, Salomon

advocated more basic research regarding innate immunity activation and regulation. Clinical applications should also employ strategies to limit tissue injury during gene delivery, and immune monitoring during clinical trials should be expanded to include assessment of innate immune markers (e.g., cytokines; chemokines; altered activation of dendritic cells, macrophages, NK cells, and downstream T or B cells).

Hildegund Ertl (University of Pennsylvania and Wistar Institute) continued the discussion by delving more deeply into the topic of adaptive immunity to viral vectors and transgene products. Her research has shown that innate immune responses are triggered in response to both human and simian replication-defective adenoviral vectors, subsequently causing a potent transgene-product–specific CD8+ T cell response that is obviously unwanted. In analyzing several AAV vector serotypes, Ertl and her colleagues found that AAV2 vectors elicit a very low adaptive immune response in animals. Preliminary analysis of liver-directed, AAV2-mediated factor IX delivery in dogs with hemophilia B proved effective, thereby prompting a phase 1/2 trial in humans with hemophilia B. Initially, one subject demonstrated transient transaminitis caused by immune-mediated liver destruction, namely, in response to the transduced hepatocytes. The trial protocol was subsequently modified to deliver less vector to participants, yet another patient still developed transient transaminitis with no detectable levels of factor IX observed. To account for these results, Ertl explained that most humans are naturally infected with AAVs that trigger the innate immune system, thereby later activating an adaptive immune response to AAV antigens and any associated helper viruses. As a consequence, humans have immunological memory to AAV that can be re-activated when the patient is exposed at a later time to AAV products, for example, the viral capsid. To overcome this problem, Ertl proposed using transient immunosuppression to prevent the loss of transduced cells, or she suggested that AAV vectors could be derived from species other than primates.

Whereas Ertl discussed the use of AAV vectors as vehicles for delivering genes recognized as "self" by the immune system, Philip Johnson (Children's Hospital of Philadelphia) discussed using AAV vectors as vaccines for delivering genetic material recognized as "foreign" and intended to produce an immune response—a situation where the objective is to elicit innate and adaptive immunity. Johnson described the use of AAV vectors in an HIV vaccine delivering the HIV-1 *gag*, protease, and a portion of the reverse transcriptase genes to cells following intramuscular injection. Preclinical trials in rhesus macaques using a human-equivalent dose range proved encouraging, with 100% of the animals producing antibody against HIV-1 Gag at 12 weeks and 100% continuing to do so at 52 weeks. Studies of the vaccine have now progressed to human (HIV-negative) clinical trials, with safety, immunogenicity, and efficacy recognized as the key outcome variables. To overcome one of the biggest hurdles—funding—in getting the trial launched, the study was initiated in Europe as opposed to the United States because a sponsor was readily available (International AIDS Vaccine Initiative) and because the study could be quickly implemented since sites were already operational. Preclinical safety assessments—specifically, of the cell substrate and the biology of the vector—also presented significant hurdles prior to trial initiation. Although the results are preliminary, short-term safety of the vaccine has been demonstrated in trial participants, and dosage-boosting studies are now being initiated.

Vaccines—Cancer and Other Diseases

Hildegund Ertl and Dale Ando (Sangamo BioSciences) co-chaired the session on hurdles faced by researchers investigating vector-based vaccines for treatment of a variety of diseases. Malcolm Brenner (Baylor College of Medicine) led off by discussing the problems associated

with using gene transfer to augment immune function—a purpose for which more than 15 investigational new drug (IND) applications have been filed. Brenner first detailed several phase 1 trials of vector-based vaccines used to elicit immune function, for example, using interleukin-2 (IL2) as a neuroblastoma tumor vaccine with either autologous or allogeneic tumor cell injections and generating a latent membrane protein 2 (LMP2)-specific cytotoxic T cell response to provide immunity against Epstein Barr virus and hence prevent lymphoma. Brenner mentioned several difficulties in bringing many phase 1 studies to trial. First, small-scale, iterative studies are often impeded by too many regulatory bodies, including governmental organizations, IRBs, lawyers, and insurance companies, among many others. Another difficulty is that pharmaceutical indifference to cell therapy, given its complexity, low profit margins, complex regulation, and poor public perception, impedes larger efficacy studies. Other problems include a lack of explicit expectations among PIs regarding their role at each stage of the clinical process and a lack of representative democracy that subsequently delays innovation—after all, the primary stakeholder is the patient, and committees should not decide on behalf of patients unless public health risks are present. To solve these problems, Brenner suggested conflating regulatory bodies by having only one local and one federal regulatory body, obtaining GMP-like manufacturing assistance for the creation of vectors to relieve some of the burden placed on PIs, and increasing patient input when making approval decisions.

Speaking next, Karin Jooss (Cell Genesys) addressed the preclinical evaluation of the granulocyte-macrophage colony stimulating factor (GM-CSF) vaccine (GVAX for short). GVAX is a genetically modified tumor cell vaccine derived from tumor cells engineered to secrete GM-CSF. At the site of vaccine injection, resident dendritic cells take up tumor antigens, process them, and present them to naïve T cells in draining lymph nodes to ultimately induce a systemic anti-tumor response. To make the GVAX vaccine as efficacious as possible, Jooss and colleagues considered the possibility that the cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which can play an inhibitory role in immunity by downregulating T cell responses, could be effectively inhibited by producing anti-CTLA4 antibodies to thereby increase the anti-tumor activity of the GVAX system. Indeed, this method proved effective in preclinical mouse and hamster studies. Despite this success, Jooss noted that 7 years lapsed between her team's initial discussion of the GVAX + anti-CTLA4 system and phase 1 enrollment of patients with hormone-refractory prostate cancer. To sidestep some of the difficulties that she encountered in getting the GVAX/anti-CTLA4 system to the clinical trial stage, Jooss acknowledged that use of murine homologs is essential for identifying safety and efficacy problems early on and that preclinical studies should use a treatment schedule similar to the one that will be used in the clinic to maintain as much consistency in the trial design and potential outcomes as possible.

John Nemunaitis (Mary Crowley Medical Research Center) discussed some of the hurdles he encountered in the product approval process during tumor vaccine trials employing adenovirus. The first obstacle was simply understanding the situation: the FDA approval process for cytotoxic chemotherapy has been established for 50 years, whereas "new" targeted therapies lack this history and are subject to much greater scrutiny. In using these new therapies optimally, the activity of the therapies should ideally be focused on the malignancy portraying the target of the therapy and should not be used in populations of patients for which only a proportion carry the target. Although several vaccines have proven efficacious in clinical trials (e.g., adenoviral ONYX-015, GVAX, other GM-CSF gene-transduced vaccines, several other oncolytic-virus-based vaccines), standard hurdles to product approval include patient accrual for trials, the focus of the grant award (basic science vs. clinical), biotech financing and protection, governance (investor focused vs. product-approval focused), profitability, and loss of insurance coverage for research participants. Nemunaitis further defined hurdles that will present

themselves as targeted therapies unfold, such as developing technologies to better define patients physiologically amenable or sensitive to the vaccine. Moreover, Nemunaitis hopes that regulatory bodies will become more accepting of "proof-of-principle" trials with regard to vector toxicity to allow researchers to move forward more quickly when delivering a different or additional gene with the same delivery vehicle.

Stephen Russell (Mayo Clinic) rounded out the session by discussing problematical issues associated with oncolytic RNA viruses. Early clinical evidence indicated that virotherapy does not usually work as a systemic therapy. Although it is still not known why the impact of intravenous administration is so limited, researchers speculate that the virus may fail to infect tumors efficiently, tumor cells could induce rapid extinction of viral gene expression, and/or viral spread may rapidly be controlled by immune responses. To address these and other issues, Russell suggested that researchers incorporate noninvasive pharmacological monitoring studies into their clinical trial designs. That is, pharmacokinetic analyses should be pursued to determine the fate of the virus in the body, and pharmacodynamic assessment should be carried out to identify how the virus works in the body. Preliminary studies with the measles virus demonstrate that it effectively infects and kills human tumor cells, and two treatment strategies using the measles virus as an oncolytic agent are currently being pursued: one using thesoluble extracellular domain of carcinoembryonic antigen as a soluble, secreted reporter for viral gene expression in patients with ovarian cancer and another using a virus expressing the thyroidal sodium iodide symporter to facilitate noninvasive imaging of viral gene expression (using radioactive iodine) in patients with multiple myeloma. Despite the promise that this technology holds, the challenges in translating measles gene transfer to the clinic include difficulties managing timeline expectations, maintaining momentum (lack of contingency funds available), getting expert help and financial support, finding a PI fully committed to the clinical process from start to finish, staffing the vector manufacturing and toxicology/pharmacology operations, learning the GMPs and GLPs, agreeing on a safe and feasible trial design, and choosing an appropriate animal model for preclinical toxicity evaluation. Moreover, the question as to who should develop intravenous virotherapy—industry or academia—remains unanswered. Although many may feel that the research is safer in the hands of academics, the capability of translating initial studies to phase 2 and 3 trials is generally lacking. However, Russell strongly advised that academic centers should develop translational capabilities to ensure that they can at least perform iterative phase I clinical testing before seeking industry partnership.

Real Stories 1

"Real stories" were presented at the Stakeholders' Meeting to provide insight into the experiences and difficulties faced by a representative set of researchers progressing through the clinical trials process. Glenn Pierce (Avigen), a co-chair of the first session of real stories along with Samuel Wadsworth (Genzyme Corp.), advised investigators involved in gene transfer studies to learn from the experiences of the presenters, and also from those in other fields such as the antibody field, so as not to reinvent history. Jean Bennett (University of Pennsylvania Scheie Eye Institute) began by discussing her experience with preclinical studies for Leber Congenital Amaurosis (LCA), an inherited retinal degenerative disease, and the successes her group has attained in correcting this defect in dogs. Bennett acknowledged the inherent difficulties in working on gene therapies for orphan diseases. Industry will rarely fund such endeavors because they stand to gain no profit since so few people have the disease. Related to this, the regulatory environment for such heavily scrutinized studies makes it increasingly difficult and expensive to get research from the bench to the bedside. Moreover, resources become fragmented when

information sharing is ignored to protect intellectual property rights. Patient selection for orphan disease trials is especially problematic since very few individuals actually have the disease of interest. To that end, Bennett recognized the need for a national database with comprehensive clinical and genetic data to identify potential participants. Preclinical ocular disease studies are further challenging because all animal models, with the exception of primates, lack a macula—the tissue of interest. Other challenges Bennett encountered are issues common to other diseases, such as the age of the subjects and stage of the disease. Because retinal photoreceptors die as LCA progresses, children represent the ideal target population for this therapy. However, informed consent issues, ethical concerns, and PI inexperience with children complicate the matter. These problems may be overcome by using only adults with advanced LCA disease in phase 1/2 studies to assess safety and efficacy and then allowing children in proof-of-principle phase 3 studies. Moreover, Bennett acknowledged a problem with heightened expectations in that the significant rescue of vision shown in dogs may not be as dramatic in humans. Consequently, patients and the general public should be educated regarding realistic expectations for the treatment.

Mark Kay (Stanford University Medical Center) presented his experience with two phase 1/2 clinical trials of AAV-mediated gene therapy for hemophilia B. Following the demonstration of safety in a phase 1/2 trial of AAV-delivered factor IX in muscle of patients with severe hemophilia B and the demonstration of safety and efficacy in preclinical dog studies employing liver-directed factor IX delivery, approval was granted for progression to human phase 1/2 studies targeting the liver. A total of 3.3 years lapsed from the IND submission until the enrollment of 7 patients due to Recombinant DNA Advisory Committee (RAC) delays stemming from an unrelated gene transfer death and disagreement between the RAC and the FDA regarding the preclinical studies. Soon after the phase 1/2 dose-escalation trial of liver-directed factor IX was finally initiated, it was halted by the FDA when AAV vector was found transiently in the semen of the first two subjects. This ultimately took 11 months to resolve. When the trial again proceeded, transient transaminitis observed in one patient led to a 9-month delay for a treatment protocol amendment followed by necessary FDA approval. Other delays were caused by patient attrition, contract negotiations between the corporate sponsor and the clinical treatment sites, and coordination of the approval process among the many regulatory bodies. These delays culminated in withdrawal of corporate sponsor support, a loss of infrastructure and momentum, and disappointment from families. Given this experience, Kay stated that he and his colleagues know what they need to do next to advance hemophilia clinical trial studies, but they are limited by prohibitive clinical trial costs and the manufacture of clinical grade vector. Kay suggested that many of the problems he encountered could be obviated with more NIH and pharma financial support during early clinical trials and by consolidating and streamlining the regulatory process.

Mark Tuszynski (University of California—San Diego) ended the first real stories session with the developmental challenges he sees with gene-based therapies for the nervous system. The nervous system represents an ideal target for gene-based therapy because a small area can be targeted with a small quantity of vector thereby resulting in less vector exposure. In addition, the central nervous system (CNS) provides partial immuno-privilege with less potential for a systemic immune response, and this area has a great unmet medical need. In contrast, vector administration to the CNS is invasive and expensive, a lack of regulation exists, outcome measures for efficacy need improved clinical scales or surrogate markers of disease activity, informed consent in dementing illness presents a unique conundrum, and control groups involving sham surgery are cumbersome and costly. Despite these factors, a number of trials of nervous system gene therapy have been initiated. To promote the advance of this field,

Tuszynski recommended that researchers initiate clinical trials based on sound efficacy and safety data from the best animal models available (e.g., primates for CNS). Moreover, the treatment design should be kept simple, such as targeting small, isolated regions with low-dose vector. Tuszynski commented that unjustified pessimism should be addressed while avoiding exaggeration, and RAC bureaucracy should be reduced since it is redundant with other regulatory agencies such as the FDA.

As a co-chair of the next real stories session, Alan Kinniburgh (National Hemophilia Foundation) began by putting forward the question, "Why is there a dearth of funding for gene therapy?" He answered that this is due to the relatively few gene therapy successes that have been achieved, the few highly publicized cases of morbidity and mortality that paint gene therapy in a bad light, and the fact that gene therapy is no longer sexy. He suggested that voluntary health advocacy groups can aid in accelerating gene therapy research by directly funding more research and by advocating for additional funding from other organizations. Moreover, put forward the possibility of creating a funding consortium to support all gene therapy research and clinical trials.

The president of the World Federation of Hemophilia, Mark Skinner, presented his agency's experience with moving gene transfer forward for hemophilia treatment. Hemophilia is typically treated by administering recombinant or plasmid-derived clotting factor. Although this therapy is effective, it is not curative and quite expensive, meaning that access is typically limited to individuals in developed countries. Consequently, the World Federation of Hemophilia adopted a goal in 1992 of promoting all research, including gene therapy, focused on finding an affordable and globally accessible cure for the disease. Many smaller hemophilia organizations have provided researchers huge monetary grants toward the same goal. Hemophilia represents an ideal disease to cure since it is well characterized genetically and clinically, there is a large market for the therapeutic product, there is a large unmet need for such treatment, and it is a simple monogenic disease with a wide therapeutic window. In reaching a cure, Kinniburgh acknowledged several areas that must be addressed. First, patient expectations must be managed so as to not elicit unfounded hope, and to that end, carefully defining what a cure will entail is important—that is, a cure may not be immediate, and physical, psychological, social, and financial burdens may not all be resolved at the same time. Second, challenges beyond basic scientific hurdles must be addressed, such as ethical concerns, informed consent issues, and the geographic variables of global clinical trials. Last, and perhaps most important, Kinniburgh stressed that achieving a cure cannot be met by the efforts of one individual, organization, company, or country; a collaborative approach is imperative.

Representing the Stop ALD Foundation, Rachel Salzman presented her organization's experience with funding gene transfer trials for adrenoleukodystrophy (ALD), a rare, X-linked, monogenic disorder that leads to elevated very long-chain fatty acids. Approximately 45% of ALD onset occurs cerebrally in young boys and causes demyelinating disease, and the remaining incidence occurs in young adults and causes peripheral neuropathy. The current ALD therapy is allogeneic stem cell transplantation, but not everyone has a match and the procedure has a 40% mortality rate. ALD gene therapy involves ex vivo correction of autologous CD34+ cells using a lentiviral vector with an ABCD1 payload (the gene responsible for X-linked ALD). Impediments to clinical gene transfer, a process in which Salzman's organization is closely involved, included challenges with the vector design and transduction protocol; fear of adverse events among investors and the public; weighing the risks versus benefits associated with safety versus efficacy; a lack of PI experience with gene therapy trials, regulatory affairs, and intimate knowledge of the ALD disease; and limited funds in addition to a limited number of scientists. Salzman's suggested solutions to these problems included forming an advisory committee of

experienced PIs, partnering clinicians with PhDs and basic researchers, investing in public relations to draw favorable attention to the research being conducted, and focusing on diseases amenable to gene therapy to garner foundation involvement for enhanced support. She concluded by stressing that orphan diseases provide a unique opportunity for treatment, because a small scientific community can speak with a united voice.

Real Stories 2

The second day of the meeting began with another round of real stories in a session co-chaired by Richard Mulligan (Children's Hospital Boston) and Cynthia Dunbar. Kenneth Fischbeck (National Institute of Neurological Disorders and Stroke/NIH) began by discussing the use of gene therapy for muscular dystrophy (MD), a disease in which muscle fibers degenerate and regenerate thereby causing progressive muscle weakness. At least 22 genes involved in MD have now been identified, and opportunities for MD therapeutic intervention entail correcting or replacing defective genes, blocking the deleterious effects of gene defects that lead to muscle degeneration, and enhancing muscle regeneration. For example, several animal and human studies have focused on replacing dystrophin, an important structural protein at the muscle plasma membrane. Although the method worked well in animal models, human studies demonstrated low gene delivery efficiency with few muscle fibers corrected. Another therapeutic approach uses antisense oligonucleotides to promote skipping of mutant or downstream exons to restore the reading frame in faulty MD genes. This method rescued dystrophin production in MD mice and cultured muscle cells from Duchenne MD patients. The only drawback is the need for individualized treatment depending on the specific gene defects requiring antisense oligonucleotide targeting. Mulligan indicated that the success of the MD therapies currently being investigated stem from the common treatment approaches taken by investigators, which facilitate an efficient use of clinical research funds and an increased chance for commercial development, especially given the connection between MD and other common diseases (e.g., age-related muscle loss).

Carl June (University of Pennsylvania) next presented the lessons he has learned from lentiviral gene transfer therapy for HIV/AIDS. HIV therapy uses a long antisense oligonucleotide targeting the HIV *env* gene encoding the viral envelope protein required for virus production and infectivity. High-efficiency transduction of primary human T cells with lentiviral vectors carrying antisense *env* has been demonstrated and suppresses HIV replication in vitro by > 2 logs. In a phase 1 trial assessing the safety and tolerability of T cells transduced with antisense *env* in 5 HIV-positive patients, no adverse events related to the protocol drug occurred and HIV viral load decreased in all patients over time, although the reduction was sometimes delayed. Moreover, the lentiviral gene transfer was sustained out to 1 year, as evidenced by the persistence of gene-modified CD4+ T cells, which suggests that multiple dosing may be possible. June explained that the major hurdles in launching the first human trials of lentiviral vectors involved regulatory obstacles (which he felt were appropriate given the potential for insertional mutagenesis with this system), patient enrollment, trying to advance to clinical trials without substantial animal model data, and ensuring institutional commitment for therapy translation. June also learned that gene therapy research funding independent of biotech is needed, because venture capital funding does not typically cover the costs of mechanism-driven studies, immunologic testing, and lifelong safety analyses that are nonetheless required in such cutting-edge trials. He also stressed the need for both empiric phase 1 trials so as not to over-rely on animal model studies, since the late-onset antiviral effects that he saw in patients would have been missed in animal studies. Another lentiviral HIV trial is currently planned—this one a

multi-dose, structured treatment interruption study—and will begin once FDA approval is attained.

Douglas Jolly (Advantagene, Inc.) related his experience in moving retroviral-based factor VIII gene transfer from preclinical to phase 1 clinical trials. Studies in animals receiving the human factor VIII retroviral vector administered by peripheral vein injection demonstrated no toxicity, boosted protein expression with higher or multiple vector-transgene doses, and prolonged factor VIII expression. For example, about 30% of dogs with hemophilia A exhibited long-term factor VIII protein expression, and almost all dogs demonstrated significantly reduced clotting times for up to 2 years, although interpretation was complicated by antibodies to the human protein in dogs. These findings enabled a phase 1 study of the treatment beginning in 1999 enrolling 13 patients with severe hemophilia A (factor VIII < 1%). Over 53 weeks, no serious adverse events occurred, no factor VIII antibodies were observed, and factor VIII expression levels ranged from 1% to 19% in 23% of all observations taken. In addressing why the vector worked well in animals but poorly in humans, Jolly surmised that the animal models were not predictive of certain prohibitive vector-human interactions (e.g., human restriction on the murine leukemia virus). Technical complications involved different vector preparations from the same packaging cell line exhibiting different properties, primarily in terms of titer. How this variability may affect preclinical or clinical outcomes is unknown. During the phase 1 study, 1 time point (out of 10 total) for 1 patient turned out positive for vector presence in semen. Although no additional positive tests appeared for this or other patients in the study, lifting the clinical hold placed on the study created a significant delay. Like many others, Jolly acknowledged that clinical research costs are substantial. He ended by arguing that extensive characterization of the vector system will promote rational responses to setbacks, which will inevitably happen. He also noted that preclinical studies are typically less expensive than clinical studies, but in the end, the clinical money must be spent; deciding when to progress to the clinical phase is the hard part.

Concluding the real stories was Ronald Crystal (Weill Medical College of Cornell University) who discussed gene therapy for Batten disease, also known as Late Infantile Neuronal Ceroid Lipofuscinoses (LINCL). This fatal, inherited disorder of the nervous system is caused by mutations in the CLN2 gene, which encodes the soluble lysosomal enzyme TriPeptidyl Peptidase 1 (TPP1) that cleaves membrane proteins to prevent their accumulation. The gene transfer strategy to treat LINCL involves delivering the normal human CLN2 gene via an AAV2 vector. Initial studies done in wild-type rats demonstrated long-term (18 months) expression of TPP1 in neurons far removed from the injection site—results then essentially repeated in non-human primates. Following a safety assessment of the clinical vector in rats and monkeys and the demonstration of restored protein cleavage following TPP1 production in the $CLN2^{-/-}$ mouse model, the decision was made to move these studies to the clinic. To date, 4 children have received the clinical vector by direct injection into the brain. Three children are stable at 7.5 or more months out, whereas 1 child died 49 days after the operation from complications due to status epilepticus. Because children are the primary targets for this therapy and given the risk of death, several ethical issues arise. To preclude some of these difficulties, the protocol is given to the families of potential participants for review, and a sincere attempt is made to dispel all therapeutic misconceptions. At the time of enrollment, the consent process is carried out by the co-investigators, the research coordinator, and a Cornell research subject advocate; the PI is not involved. Like other orphan diseases, Batten disease falls into a funding gap between NIH support and venture capital/pharma monies. The total costs of the clinical study are estimated at $4 million over 3 years. As a result, funding provided by various foundations (i.e., NIH PI funding, Department of Genetic Medicine, and Nathan's Battle

Foundation) is critical for enabling the research to get done. However, Crystal noted that donating foundations are divorced from control over the clinical research to safeguard against personal motives and to promote the greater good of all.

FDA/NIH Perspectives

The last of the sessions, co-chaired by Stephanie Simek (Office of Cellular, Tissue, and Gene Therapies [OCTGT]/Center for Biologics Evaluation and Research [CBER]) and Kenneth Cornetta (Indiana University/National Gene Vector Laboratory [NGVL]), centered on the FDA/NIH's perspective regarding the challenges in advancing the field of gene therapy. Simek prefaced the session by indicating that although the number of active gene therapy INDs in later phase trials are few in number—more than 160 in phase 1 versus slightly more than 60 in phase 2 and only about 5 in phase 3—the FDA is strongly committed to moving the field forward. She laid out a number of questions focused on safety and efficacy that all parties should keep in mind when devising how to advance their gene transfer research (Table 1). Furthermore, she stressed that academic researchers should remain involved in the transition to phase 3 trials given their wealth of knowledge about the basic gene delivery system that must be carried forward—knowledge that becomes critical if the project encounters a problem and needs to be stepped back to the phase 1/2 level for further refinement.

Table 1. Questions Researchers Should Consider to Advance Gene Transfer Research

- In an ongoing phase 1 clinical trial of a specific gene therapy product, what product changes would require a new IND versus performing a preclinical bridging study to demonstrate safety?
- If the product changes prompt a sponsor to perform a comparability study,
  - what type of testing should be included in the study?
  - how should the study be conducted?
    - Must a direct, side-by-side comparison of the old and new products be done?
    - Can data from previous lots of old product be used?
- What type of assay would the FDA accept for use as a measure of product potency?
  - What is the importance/benefit of having a quantitative potency assay?
  - Will the agency accept a qualitative assay if a potency assay cannot be quantified?
- Do animal studies need to be conducted with the product?
  - Do vector biodistribution studies need to be conducted if there is published data with this type of vector?
  - Do vector toxicology studies need to be conducted if there is published data with this type of vector?
- What is an appropriate animal model for the vector of study?
  - Must non-human primates be used?
  - Can analogous vectors or transgenes be used in species other than non-human primates?
  - Does the agency accept data in animal models of disease for demonstration of activity? Safety?
  - Do all animal studies need to be GLP-compliant?
- At what point in time should CBER be contacted to discuss preclinical studies?
- What types of control groups might be needed for conduct of "adequate and well controlled" clinical studies to support registration for a gene transfer product?
- Hoe does the FDA determine a study's designation (i.e., phase 1, 1/2, 2, or 3)? Does the FDA have specific criteria for making this designation?
- Does the FDA accept clinical study data from trials conducted in countries outside of the US?
- What advantages does the "fast track" development program designation confer to sponsors?

Daniel Rosenblum (OCTGT/FDA) led off the trio of speakers with his perspective on the preclinical and clinical challenges of gene transfer studies. He stressed that safety represents the primary factor for improving the prospects for gene transfer success. In conjunction with this, he suggested that researchers seek FDA advice and keep the organization informed regarding changes in research plans, be able to adapt strategies to account for new developments, characterize the product mechanism of action to facilitate bringing the product to licensure, begin with a label in mind, and maintain focus throughout it all. He stated, "The first step in

developing products for clinical use is to establish that they are reasonably safe to test in humans." To that end, the FDA relies heavily on relevant preclinical data to make the assessment regarding a safe starting clinical dose, a safe scheme for dose escalation, and whether a clinical benefit can be obtained without excessive toxicity. The FDA also carefully examines the scientific basis for the clinical study design to verify that the rationale is sound, elements of the protocol have established data to back them, and adequate preclinical data are available to support the proposed clinical trial design. The FDA exists to safeguard against excessive patient toxicity that may occur during phase 1/2 exploratory studies. Hence, all phase 1/2 designs should define the optimal population that is predicted to have the largest clinical benefit at the smallest risk. Phase 2/3 confirmatory studies should be designed with the objective of demonstrating efficacy.

Andrew Byrnes (CBER/FDA) continued this theme by specifically focusing on common challenges in the development of gene therapy products. He stated that researchers are not ready to proceed to pivotal clinical trials until the product has been adequately characterized. Product characterization entails undertaking specific tests to demonstrate product consistency between lots and assuring comparability after manufacturing changes. Above all, product characterization should demonstrate (a) correct identification of the product to verify that the vial contents match the label, (b) potency of the product based on a unique assay to measure biological function (ideally a quantitative measure of bioactivity; e.g., a measure of oncolytic adenovirus viral replication), and (c) product stability throughout all phases of the production process, which can be used to determine expiration dating and shipping and storing conditions. To quell any collective groan from audience members, Byrnes argued that good product development does not just help to get products passed by the FDA; it also can aid in product development and the clinical trial process by generating solid data. In contrast, not properly characterizing one's product may lead to difficulty in attracting partners and investors, and pivotal trials may be placed on hold until such analyses are carried out. Byrnes further advocated for product characterization by arguing that it is better to uncover problems sooner rather than later, the transition to pivotal trials and commercialization will be easier, a poorly characterized product may lead to unpredictable clinical results, and the expense of product characterization is small compared to the expense of repeating a clinical trial. To succeed at product characterization, the product and manufacturing process should be designed with consistency in mind at the very start, and product characterization should be initiated early.

The last of the presenters, Kenneth Cornetta, discussed clinical trial challenges from the perspective of the NGVL. Formed roughly 10 years ago, Cornetta explained that the NGVL is an interactive, NIH-sponsored group with the goal of supplying investigators with clinical-grade vectors and toxicology support for gene therapy applications. Major lessons that Cornetta has learned in the past several years are that (a) cell lines generated in investigators' laboratories often do not meet quality standards and (b) investigators are struggling with grant timelines and regulatory issues related to adverse events that stifle the progress of many trials. Cornetta observed that scientists also face many challenges stemming from the rapidly evolving field of vectorology. He mused that "today's hot vector is tomorrow's dinosaur," which limits the availability of guidance documents and tacks on time to the vector production process due to new production and certification assays. In addition, investigators are strapped by financial difficulties owing to limited NIH support that does not last through the many review board hurdles PIs must overcome before clinical trials gain approval; some investigators may need three or more sources of funding for a single trial. PIs also face toxicology challenges since the wide variety of viral vectors makes standardized testing difficult and because traditional toxicology studies are not ideally suited to study biologicals. On the positive side, Cornetta

noted that the science and vectors keep getting better. Moreover, the NIH has had outstanding advocates for research and patient safety, the FDA and scientists from academia have been extremely dedicated to getting the research done, and academic institutions have made major investments in fostering clinical gene therapy.

Following the conclusion of the talks, additional panelists—Maritza McIntyre (CBER/FDA) and Mercedes Serabian (OCTGT)—joined the session participants for a general discussion with meeting participants. One issue that emerged was that, although not traditional, academic institutions can perform phase 3 trials and take a product all the way to licensure if they so choose; biotech/pharma control is not required. Some participants took issue with the fact that phase 1 trials often administer sub-therapeutic doses to patients. The panelists argued that there are ways to design safety trials where sub-therapeutic doses are not administered to all patients. The FDA also indicated that intra-patient dose escalation is a possibility if investigators can prove that the escalation will be safe. One participant asked whether there is any interaction between U.S. and European regulatory bodies to harmonize their regulations, as this becomes an issue for multi-center trials with European partners. The FDA noted that European participants in U.S.-sponsored trials have to comply with FDA requirements just like all U.S. participants. Returning to a common grievance, someone from the audience asked, "No one doubts the importance of regulations, but is there a way to make the process more streamlined for faster production?" For example, the role of the RAC is a source of contention for many researchers, because although it is not an official regulatory body, many IRBs regard it as such, and PIs are forced to follow suit. It was noted that FDA reporting has become more streamlined since the NIH Genetic Modification Clinical Research Information System (GeMCRIS) now allows online filing of reports, and the FDA is also willing to work with investigators when protocol changes are made so that researchers need not necessarily return to square 1 if they can empirically demonstrate that the protocol changes will not affect patient safety.

Conclusion

The concluding portion of the meeting was co-chaired by the co-moderators of the conference, Salomon and High. Salomon recapped issues discussed throughout the meeting by presenters and in conversations following the sessions, emphasizing above all the approaches that will enable the gene therapy field to move forward in clinical trials. To move vectorology forward, Salomon recapitulated that new strategies should be developed to direct integration safely, more attempts should be made to deliver or repair genes without insertional events, gene expression requires better regulation and tissue specificity, and the process of sorting out the best applications for specific vectors must continue. Moreover, he noted that it is critical for academics to approach the gene transfer "product" in the context of the entire process from phase 1 to phase 3 to facilitate biotech and pharma support. Whereas some audience members agreed that scientists need to have the end product in mind to garner funding, others felt this is an inefficient and unnecessary approach since pharma will likely alter the basic characterization of the product to tailor it to their methodology.

In turning to immunology, Salomon commented that immunity should not be viewed as an insurmountable barrier but just as a challenge. To move immunology forward, he noted the potential for developing therapies that do not activate the innate immune response and the use of immunosuppressive therapy, ideally only short term, to repress the innate and adaptive immune responses. More headway can be gained in this area if scientists team with immunologists to overcome immunological problems due to gene transfer.

Major progress has been made in the area of gene transfer vaccines, although persisting uncertainty regarding the selection of appropriate animal models requires resolution and more rigorous pharmacokinetic and pharmacodynamic analyses of gene transfer systems are needed.

In turning to the important topic of the regulatory climate, Salomon observed that multiple players in the evaluation and approval process for clinical trials, each with perfectly appropriate but intensely held views of what is best for the many different stakeholders involved, are actually creating disincentives, delays, and significant obstacles to successfully initiating clinical trials. Everyone agreed that this is not the intention of any group involved and that the whole environment in the United States for the review, approval, and conduct of clinical trials is difficult. In other words, all invested parties have to be careful not to assume that every obstacle is unique to gene therapy. In trying to address these issues, Salomon argued that the best current target for refining the regulatory process involves establishing a constructive dialog with the local IRBs on a national level; however, this can only be done with great sensitivity and must fully respect IRB autonomy and each's special mandate for protecting research subjects and insuring ethical conduct of clinical research. An IRB director in the audience made the salient point that the mission of IRBs does not require justifying their process or their decisions to the physician investigators. Nonetheless, there was a consensus that everyone involved, especially the patients, have a major stake in supporting the success of clinical research. Thus, the ASGT should begin to reach out to IRBs and offer to provide necessary scientific expertise and support when requested in ways that improve the process. However, it is equally important that investigators begin to determine and understand the concerns of the IRBs with applications for clinical trials in gene therapy and work to address these issues proactively with their colleagues.

Other strategies for advancing the field involve improving public perception of gene therapy through education and public outreach efforts. Indeed, since the workshop in April 2005, ASGT has launched a new committee to take on this task. Another need is to increase the level of experience at academic centers regarding clinical trial conduct to support and train the next generation of investigators.

A major sticking point—and one of the most commonly heard grievances at the meeting—involves the issue of funding. First, the focus of pharma on "big markets" creates a funding gap for many current gene therapy targets aimed at relatively small "markets" such as single-gene-defect diseases. This lack of enthusiasm from pharma, albeit understandable in some terms, hinders translation of clinical gene therapy. One line of discussion was that some gene therapy should take on approaches in the major markets like cardiovascular disease, obesity, and diabetes. It was also pointed out that a significant amount of work in gene therapy is now directed at cancer, which is certainly a big market area. Proof of the success of gene therapy in a small-market disease would effectively bring about the necessary attention of pharma. In fact, a venture capital investor in the audience emphasized this point and noted how these issues of success perception were negatively impacting investment in small biotech companies for gene therapy. The discussion also considered the value of academic investigators taking more responsibility for starting projects with a more pragmatic development plan that considers issues to be encountered at later stages of production and implementation for clinical trials. The argument was made that a better plan from the very initiation would position academic advances for much easier acceptance by both small biotech and big pharma when success is demonstrated at the early phase 1/2 stage. However, others argued that this may be difficult to consistently execute when working with cutting-edge technologies where a clear view of later manufacturing and implementation issues is impossible. Finally, the NIH was recognized and applauded for its pivotal role in funding the development of gene therapy and supporting clinical trials through resources such as the NGVL and the NCI's Rapid Access to Intervention Development (RAID)

program. However, it was noted that the technical structure of classic NIH funding does not always mesh well with the timelines faced by researchers trudging through the regulatory process. This may limit early-phase research, reduce institutional interest in supporting this critical but risky stage, and narrow the field, particularly by serving as a major disincentive for young investigators with few of their own resources. A constructive suggestion was to create a more flexible funding structure for clinical trials as well as strategies to support investigators in dealing with the process of planning and approvals. In fact, this strategy is currently in development as part of the NIH Director's Roadmap.

In the final analysis, all workshop participants agreed that there has clearly been significant progress in gene therapy in the last few years, and there remains strong support for the field in many areas. The recent advances in anti-tumor vaccines and in delivery of genes with new vector designs suggest that the gene therapy field is gaining momentum. Identification and mechanistic understandings of the problems the field now faces in vectorology, insertional mutagenesis, and immunity are necessary first steps to designing strategies to successfully address these challenges. In the meantime, the consensus was that the ASGT should work on two areas: (a) educating the public about the successes of and significant progress in gene therapy while ensuring that the failures are not unreasonably exaggerated, and (b) working with all involved stakeholders in the approval and review of clinical trial protocols to improve the process and reduce the disincentives currently recognized.

Many of the summary slides for this workshop can be found on the ASGT web site: www.asgt.org.

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Appendix C

Viscomi, "Structure-activity of type I Interferons," Biotherapy 10:59-86 (1997)

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Structure-activity of type I interferons

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Abstract

Type I IFNs consitute a family of proteins exhibiting high homology in primary, secondary, and tertiary structures. They interact with the same receptor and transmit signals to cellular nucleus through a similar mechanism, eliciting roughly homogeneous biological activity. Nevertheless, the members of that family, IFNα species, IFNβ and IFNω, due to local differences in the structure sometime show distinct properties. From the reported data it results that even minute changes or differences in the primary sequences could be responsible for a significant variety of biological actions, thus inducing to the hypothesis that Type I IFNs, rather than to be the result of a redundant replication during the evolution, play definite roles in the defense of living organisms to foreign agents.

Abbreviations: Ab-IFNα – antibodies to alpha interferon; Ab-IFNβ – antibodies to beta interferon; Ala – alanine; Arg – arginine; Asp – aspartic acid; Bo – bovine; Cys – cysteine; Gln – glutamine; Glu – glutamic acid; Gly – glycine; His – histidine; Hu – human; IFN – interferon; IFNα – alpha interferon; IFNβ – beta interferon; IFNγ – gamma interferon; IFNω – omega interferon; IFNτ – tau interferon; IFNA – gene of alpha interferon; IFNB – gene of beta interferon; IFNW – gene of omega interferon; Ile – isoleucine; Jak – Janus kinase; LE-IFNα – leukocyte alpha interferon; Leu – leucine; LY-IFNα – lymphoblastoid alpha interferon; Lys – lysine; mAb – monoclonal antibody; Met – methionine; Mu – murine; N-Ab-IFNα – antibodies to alpha interferon neutralizing in vitro antiviral activity of alpha interferon; NN-Ab – antibodies to alpha interferon non neutralizing in vitro antiviral activity of alpha interferon; NN-Ab-IFNβ – antibodies to beta interferon non neutralizing in vitro antiviral activity of beta interferon; Pab – polyclonal antibody; Phe – phenylalanine; poly I:C – polyinosinic:cytidylic acid; Pro – proline; rIFNα – recombinant alpha interferon; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ser – serine; Thr – threonine; Trp – tryptophan; Tyk – tyrosine kinase; Tyr – tyrosine; Val – valine.

Introduction

The interferons (IFNs) among cytokines consitute the most complex family. A number of IFNs have already been identified, although further discoveries cannot be excluded. Five type of interferon are currently distinguished: IFN alpha (IFNα – also known as leukocyte interferon), IFN beta (IFNβ – also known as fibroblast interferon), IFN gamma (IFNγ – also known as immune interferon), IFN omega (IFNω), and IFN tau (IFNτ – also known as trophoblastic factor) [1, 2].

They exert their activity through an interaction with membrane receptors [3, 4, 5]. IFNα, IFNβ, and IFNω bind to the same receptor, the so-called type I, which is composed of two subunits recently cloned [6, 7], modulating biological effects *in vitro*, such as establishing antiviral status, inducing antiproliferative and immunostimulating activities [8, 9, 10]; IFNγ interacts with a specific receptor, the so-called type II, and it eminently shows immunomodulating activity [3, 11]. IFNτ diverge from the above mentioned, since it is biologically active during the embryo preimplantation of ruminants. In the present report attention will be focused only on Type I IFNs.

To date, 13 genes and 5 pseudogenes of IFNα, 1 gene and 6 pseudogenes of IFNω, and 1 gene of IFNβ have been identified. All these genes are located on the short arm of chromosome 9, at band 9p21. They can be

considered belonging to two groups indicated as distal and proximal. The distal group has a more recent origin and contains genes IFNA21, IFNA4, IFNA7, IFNA10, IFNA16, IFNA17, IFNA14, IFNW1, and pseudogenes IFNWP15, IFNWP9, IFNWP18, and IFNWP5. The IFNB gene is located at the end of this distal group. The proximal group, expanded earlier in the evolution, contains genes IFNA5, IFNA6, IFNA13, IFNA2, IFNA8, IFNA1, IFNW, and pseudogenes IFNAP22, IFNAP20, IFNAP11, IFNAP12, IFNAP23, pseudogenes IFNWP2, and IFNWP19. IFNA genes within the distal group are more closely related than the IFNA genes of the proximal group [12].

The Type I IFN family contains proteins of 166 amino acidic residues, except that IFNα2 which has 165 residues, and IFNω with 172 residues.

The apparent molecular weights range between 16000 and 28000 D, when measured in SDS PAGE. Only some of them are glycosylated, namely IFNα2 is O-glycosilated on Tyr 106, IFNα14 is N-glycosylated on Asn 2 and Asn 72, IFNβ on Asn 80, and IFNω on Asn 78.

All the IFNα subtypes and IFNω contain two disulfide bridges at the position Cys 1-Cys 99, and Cys 29-Cys 139, except that IFNα2 which lacking position 44 forms the disulfide bridges with Cys 1-Cys 98 and Cys 29-Cys 138, and for IFNα8 which forms the first disulfide bridge with Cys 1-Cys 100. IFNβ has only one disulfide bridge at Cys 31-Cys 141.

The reason for the natural existence of such cospicuous numbers of IFN species which act on the same receptor is still under investigation; from the reported data unequal biological activity of the various IFN species could be inferred. They showed different antiviral activity [13, 14]; different stimulation of the natural killer activity (NK), in which some subtypes, like IFNα7, act as antagonist of other ones, like IFNα2 [15, 16]; different stimulation in monocytes of the antigen of the major histocompability complex [17, 18]; different inhibitory ability of cell growth [19, 20, 21]; and different interaction with IFN receptors in cells of different animal species [22].

From the interaction with the receptor emerges a signal transduction similar to that of most of cytokines. The binding proteins of the IFNα/β receptor do not contain any functional enzyme but recruit cytoplasmic tyrosine kinases among them of the Janus family, namely Tyk2 and Jak1 [23].

The Type I IFNs have 33 completely conserved positions. The subtypes of IFNα, in particular, have the highest homology, up to 95% for the couples IFNα4/IFNα7, IFNα4/IFNα17, IFNα5/IFNα22, which correspond to 7–9 different residues. While the lowest homology in IFNα subtypes (78%) is present for the couples IFNα1/IFNα7 and IFNα1/ IFNα8 corresponding to differences in 35–38 residues. Structurally closest to IFNβ are IFNα2 and IFNα22 with 33% of homology, while the minimal homology (29%) is shown by IFNα1, IFNα8, and IFNα21. Both IFNβ and IFNω, when compared to IFNα subtypes, show the highest homology to IFNα2 [24].

Therefore, the members of Type I IFNα family consist of molecules with a variable homology, from 29% to 95%, interacting with the same receptor and eliciting similar biologial affects. Nevertheless, since differentiated biological activity has been recognized some fine differences should be present among Type I IFNs in the binding to the receptor and transmitting the signal.

The aim of the present paper is to report data relative to structure-activity of Type I IFNs with the intent to show that some different properties may be attributed to structural reasons.

The hypothesis is that all the molecules of Type I IFNs retain a common structure, but due to local differences in the primary sequence they diverge in some way in the interaction to the receptor, thus inducing modulated or differentiated effects.

Structural and functional data of Type I IFNs

Based on a computer-building approach, a three-dimensional model for human Type I IFNs has been constructed [25], challenging the results with X-ray crystallography data available for murine IFNβ (Mu-IFNβ) [26]. From this representation the tertiary structure of Type I IFN consists of five major α-helix segments indicated as Helices A (encompassing the sequence 12–24), Helix B (51–67), Helix C (80–99), Helix D (115–132), and Helix E (141–165), which are arranged up-up-down-up-down. The interconnecting regions are designed Loops AB (25–50), BC (68–79), CD (100–114), and DE (133–140). A short helix structure may be recognized in Loop CD, too. Similar results have been obtained by other authors, even if some minor differences are evidenced in the predicted locations of helices and loops [24, 27].

Large number of data are reviewed on the structure–activity relationship of Type I IFNs. Unfortunately this information is concentrated mainly on a few species, namely IFNα1, IFNα2, IFNα4, and IFNβ.

Reduced amount of data are available for IFNα8, IFNα7, IFNα21, and IFNω. Only rare information can be collected for the remaining components of the family.

The data are reported in Table 1, following the order of the sequence position, to which they are referred (first column). The shadowed areas of Table 1 represent conserved positions at least between IFNα subtype.

As reference, the sequence of IFNα2b has been used, where an extra position at 44 has been introduced (second column). When data of IFNβ and IFNω are dealt with, the position number corresponds always to that of IFNα2b, after having properly aligned the sequences of IFNβ and IFNω to get the best fit in the homology [24].

The choice of such classification is based on the conviction that it could be useful to compare together disparate information concerning the same sequence position (third column).

The data of Table 1 represent an incomplete collection; nevertheless, the hope is that they are enough to show how complex is the matter, to give some interpretation-keys for further data and, finally, to permit some partial conclusions.

Discussion

The data of Table 1 will be discussed only with the intent to draw some general statements, leaving to the reader the opportunity to interpret them in more detail. The distribution of critical positions along Type I IFN sequences suggests several regions of the IFN molecules are involved in eliciting the biological activity, and the evolutional differentiation, that produced the members of Type I IFN family, acted over the entire sequence as consequence of the need to follow IFN receptor modifications, to create or to maintain the specificity over different animal cells or over histologically different cells, and to specialize the biological activity of some of them.

This hypothesis is confirmed by the observation that fragments of Type I IFN sequences, obtained by synthesis or proteolytic cleavages, were never able to display or inhibit the entire biological activity of IFNs [29, 30, 31, 32, 33]. In this context it is interesting to note that the region of IFNα with the highest homology of sequence, namely 140–150, is poorly involved in the biological activity; needless to say that this region has not been differentiated during evolution, since it is not relevant.

Type I IFNs have two main binding sites to IFN receptor, one at high affinity responsible for the binding to the receptor, an other one at lower affinity involved in mediating signal transduction [34, 35, 36, 37]. These two binding sites are spatially distinct, in the first one Helices A and B, and Loop AB are involved with a large contact area, while the second one engages Helices A and C, and Loop DE in a smaller contact area. Therefore, at least based on that description two distincted fragments should be needed for eliciting the biological activity.

Many positions of Type I IFNα have been recognized as important for the receptor interactions. Continuous areas formed by charged residues, which form electrostatic potentials complementary to receptor potentials, have been hypothesized. For example, in the case of IFNα8 a positively charged area is assumed to be composed of the residues at positions 12, 13, 22, 23, 31, 33, 34, 46, 50, 145, 150, 160, and 165. A negatively charged zone is assumed to be formed by residues 78, 79, 83, 84, 88, 90, 95, and 97. While an area suitable for hydrophobic interaction is assumed to be arranged by residue 9, 16, 17, 30, 152, and 154 [37].

On the basis of structural analysis of Type I IFNs and IFN receptor, the positions that are believed to be directly in contact with the receptor are 6, 7, 10, 13, 14, 17, 18, 20, 21, 27, 28, 30, 31, 33, and 43 [24, 27, 37, 38, 39, 40].

Furthermore, from Table 1 it results that crucial roles are also played by some residues, which are functionally important to confer the proper structure to the IFN molecules. The two Cys residues, forming disulfide bridge 29–139, are critical. The inhibition of the formation of this disulfide bridge or just the displacement of its location of only one position causes loss of activity [41].

The residues of IFNα2 Phe 36, Leu 67, Trp 141, and Val 143 form a hydrophobic pocket surrounding the side chain of Leu 131. The substitution of Phe 36, Trp 141, and Leu 131 has been demonstrated to abrogate the activity, thus showing how important is this structural motif [37].

Tyr 123 seems to act as spacer between Helix D and Loop AB. The substitution of this residue with aromatic residues, like Phe or Trp, has a minor effect on the biologically activity, while the substitution with charged and/or hydrophillic residues produced a dramatic decrease of the antiviral effect [42].

A similar role is played by Tyr 130, which appears to be a spacer between Helix D and Loop AB [42].

Table 1.

No.	Residue	Note	Ref.
1	Cys	Conserved residue in the IFNα subtypes and IFNβ	
1	Cys	IFNα1 analogue with Ser 1 completely maintained the biological activity	[41]
1	Cys	Cys involved in the disulfide bridge 1–99, which is not essential for the biological activity	[79]
1	Cys	The anologue of IFNα1 with the substitution of Cys 1, 86, and 99 with Ser residues retained 23% of biological activity in human cells, but the activity was not affected on bovine cells	[119]
1–4		Truncated analogue of IFNα2 retained the antiviral, antiproliferative, and immunostimulating activity	[41]
1–5		Truncated analogue of IFNα2 retained the antiviral, antiproliferative, and immunostimulating activity	[41, 81, 82]
1–10		Truncated IFNα2 had poorly detectable antiviral effect	[41]
1–11		Replacement of that fragment of IFNα2 with the corresponding to IFNα1 produced an IFNα2 analogue with similar biological activity respect to the IFNα2	[41]
1–12		Truncated IFNα2 had poorly detectable antiviral effect	[41]
1–14		Truncated IFNα2 was inactive in the antiviral activity	[41]
1–16		Truncated IFNα2 had poorly detectable antiviral effect	[41]
1–21		Truncated IFNα2 did not have antiviral effect	[41]
1–21		Deletions in this region inactivated IFNα2, but not the NK– 2 binding to the protein.	[41, 83]
1–23		mAb, named 1-4-A, to that fragment of IFNα4 inhibits signal transduction, but not receptor binding	[71]
1–29		Deletions in this region inactivated IFNα2 but not the NK– 2 binding to the protein.	[41, 83]
1–60		Hybrid [α8(1–60), α1(61–166)] had close optimal activity on human cells and good activity on murine cells.	
2–7		Replacement of that segment of IFNα2 (Asp-Leu-Pro-Gln- Thr His) by the unrelated IFNγ sequence (Tyr-Cys-Gln-Asp-Pro-Tyr) did not affect the activity	[41]
3	Leu	Conserved residue in IFNα subtypes, IFNβ, and IFNω	
5	Gln	mAb, designed U1, binds to region 5–15 of IFNα2 but not at all to IFNα1. The differences in the sequence are at the position 5(Gln in IFNα2, Glu in IFNα1), 10 (Gly in IFNα2, Asp in IFNα1), and 11(Ser in IFNα2, Asn in IFNα1)	[75]
5	Gln	The [Glu 5, Ser 27, Met 31, Leu 60] IFNα2 analogue containing four residues of IFNα1, which do not occur in those positions in any other IFNα subtypes, had antiviral potency close to that of IFNα1	[41]
5	Gln	The [Glu 5, Asp 10] IFNα2 with the modified residues corresponding to those of IFNα1 was biologically and immunologically similar to IFNα2	[41]
6	Thr	Conserved residue in the IFNα subtypes	
6	Thr	This position is predicted to be involved in the interaction IFNα8-receptor (D200′ model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
7	His	Conserved residue in the IFNα subtypes and IFNω	
7	His	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
7	His	This position is predicted to be involved in the interaction IFNα8-receptor (D200′ model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
9	Leu	Conserved residue in the IFNα subtypes and IFNω	

Table 1. cont.

9	Leu	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	[37]
9–18		The synthetic peptide corresponding to IFNα cons sequence inhibited both the proliferation of the Daudi cell and the antigen receptor-stimulated proliferaton of fresh human T lymphocytes.	[84]
10	Gly	This position is predicted to be involved in the interaction IFNα8-receptor (D200′ model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
10	Gly	mAb, designed U1, binds to region 5–15 of IFNα2 but not at all to IFNα1. The differences in the sequence are at the position 5(Gln in IFNα2, Glu in IFNα1), 10 (Gly in IFNα2, Asp in IFNα1), and 11(Ser in IFNα2, Asn in IFNα1)	[75]
10	Gly	The [Glu 5, Asp 10] IFNα2 with the modified residues corresponding to those of IFNα1 was biologically and immunologically similar to IFNα2	[41]
10	Gly	Replacement of all five Gly residues of IFNα2 with chiral residues like Ala residues produced analogue [Ala 10, 37, 45, 103, 105] IFNα2, which is indistinguishable from IFNα2	[41]
10	Gly	It is the most critical residue in the epitope of mAb-I-4-A to explain the selective reaction to subtype IFNα4a and IFNα2b, but not IFNα1 and IFNα14	[71]
10–15		Replacement of that fragment of IFNα2 with five Ala residues produced a 100-fold reduction in the biological activity.	[41]
10–44		Bovine IFNαC is inactive on human cell, and the replacement of the sequence 10–44 by corresponding sequence of human IFNα7 produced an hybrid that was partially active on human cells. In that fragment at least five of the following ten positions are considered critical: 21, 22, 24, 27, 31, 34, 35, 37, 40, 42, and 43.	[40]
11	Ser	mAb, designed U1, binds to region 5–15 of IFNα2 but not at all to IFNα1. The differences in the sequence are at the position 5(Gln in IFNα2, Glu in IFNα1), 10 (Gly in IFNα2, Asp in IFNα1), and 11(Ser in IFNα2, Asn in IFNα1)	[75]
12	Arg	Conserved residue in the IFNα subtypes and IFNω	
12	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
13	Arg	Conserved residue in the IFNα subtypes	
13	Arg	Ifnar1 would "see" Arg 13 of IFNα2	[25, 27]
13	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
13	Arg	This position is predicted to be involved in the interaction IFNα8-receptor (D200′ model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
13	Arg	The residues Arg 13 and Leu 17 are predicted to be also involved in the IFNα8-p40 model at site 1	[37]
14	Thr	Ifnar1 would "see" Thr 14 of IFNα2	[25, 27]
14	Thr	This position is predicted to be involved in the interaction IFNα8-receptor (D200′ model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
16	Met	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	[37]
16	Met	Single mutation of each of Met 16, 21, 60, 112, and 149 of IFNα2 with Leu residues, and contemporary substitution of all Met residues with Leu residues produced analogues indistiguishable from unmodified IFNα2	[41]
16–29		This region contains elements responsible for the reduced activity of IFNα1 on human cells	[40]
16–29		That sequence is responsible for IFNα1 being only partially active on human cells	[45]
17	Leu	Ifnar1 would "see" Leu 17 of IFNα2	[25, 27]

Table 1. cont.

17	Leu	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	[37]
17	Leu	This position is predicted to be involved in the interaction IFNα8-receptor (D200' model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
17	Leu	The residues Arg 13 and Leu 17 are predicted to be also involved in the IFNα8-p40 model at site 1	[37]
18	Leu	This position is predicted to be involved in the interaction IFNα8-receptor (D200' model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
18–45		mAb to that fragment of IFNβ does not neutralized the antiviral activity	[70]
20	Gln	Conserved residue in the IFNα subtypes and IFNω	
20	Gln	Ifnar1 would "see" Gln 20 of IFNα2	[25, 27]
21	Met	Conserved residue in the IFNα subtypes and IFNω	
21	Met	Ifnar1 would "see" Met 21 of IFNα2	[25, 27]
21	Met	This position is predicted to be involved in the interaction IFNα8-receptor (D200' model at site 2). The residues involved are: Thr 6, His 7, Gly 10, Arg 13, Ala 14, Leu 17, Leu 18, Met 21	[37]
21	Met	Single mutation of each of Met 16, 21, 60, 112, and 149 of IFNα2 with Leu residues, and contemporary substitution of all Met residues with Leu residues produced analogues indistiguishable from unmodified IFNα2.	[41]
22	Arg	Site-direct mutagenesis of Arg 22 and Arg 33 of human IFNα2c with Lys and His respectively produced an analogue with antiviral activity on human and bovine cells of 2000%, and 124%, respectively	[43]
22	Arg	At least one of residues 22, 26, and 27 should have strong effect on antiviral activity on human cells	[45]
22	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
23	Arg	Alteration of Arg with Lys in that position (IFNα2a/b) did not affect its biologic activity, but modified antigenic structure in the N-Terminal domain	[85, 86]
23	Arg	The neutralizing antibodies which contained in their epitopes that position inhibited the binding of IFNα2b to human cells.	[86, 87]
23	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
23	Arg	The residues at the positions 23 and 34 are predicted to be exposed and sterically accessible	[88]
24	Ile	Conserved residue in the IFNα subtypes and IFNω	
24–29		The residues of this sequence are proposed to interact directly with ifnar1	[38]
25	Ser	Conserved residue in the IFNα subtypes and IFNω	
25–28		The sequence of human IFNα1 is identical to that of the bovine IFNα, where it is a conserved sequence	[45]
26	Leu	Single replacement in that position had only minor or insignificant effects on antiviral activity of IFNα4	[24]
26	Leu	At least one of residues 22, 26, and 27 should have strong effect on antiviral activity on human cells	[45]
26–37		Changes of the antiviral activity due to modifications in this region of IFNα4 was accompained by similar changes in the antiproliferative activity	[89]
27	Phe	Single replacement in that position significantly contributed to low specific activity of IFNα1	[41]
27	Phe	Single replacements in that position had only minor or insignificant effects on antiviral activity of IFNα4	[24]

Table 1. cont.

27	Phe	A large hydrophobic residue is found in that position in all human and murine IFNα except that in IFNα1. This kind of residue appears to be necessary for full activity on human but not on bovine cells	[45]
27	Phe	Large hydrophobic residue appears to be necesary for full activity on human cells but not bovine cells	[38]
27	Phe	Ligand lacking of a hydrophobic residue at the position 27 and having Met 31 instead of Lys (common situation in all the human and murine IFNs) produced an inactive complex with bovine ifnar1/murine ifnar2	[38]
27	Phe	The [Glu 5, Ser 27, Met 31, Leu 60] IFNα2 analogue containing four residues of IFNα1, which do not occur in those positions in any other IFNα subtypes, had antiviral potency close to that of IFNα1	[41]
27	Leu	At least one of residues 22, 26, and 27 should have strong effect on antiviral activity on human cells	[45]
28	Ser	Conserved residue in the IFNα subtypes	
28	Ser	[Cys 28, Ser 29] IFNα2 analogue, where the sequence of the position 28 and 29 are inverted, was devoid of biological activity on human cells	[41]
29	Cys	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
29	Cys	Importan disulfide bridge	[46, 90]
29	Cys	[Ser 29, Ser 139] IFNα2 showed reduced but still detectable antiviral activiy.	[41]
29	Cys	[Gly 29, Gly139] IFNα2 was inactive	[41]
29	Cys	[Leu 29, Cys 30] IFNα2, where the sequence of the position 29 and 30 are inverted, was devoid of biological activity on human cells	[41]
29	Cys	The [Cys 27, Phe 28, Ser 29] IFNα2 analogue with the displacement of Cys 29 by two position down along the chain was biologically inactive	[41]
29–33		The residues flanking that region have been shown to influence the presentation of that sequence	[76]
29–33		The [Cys 29, Ala 30, Lys 31, Ala 32, Ala 33] IFNα2 mutant was inactive on both the bovine and human cells	[91]
29–35		The domain is in close spatial proximity to the fragment 123–140 in the proposed model. All the residues are accessible.	[25]
29–36		Policlonal antibodies to that fragment reacted well with IFNα1 and IFNα4, but weakly with IFNα2	[75]
29–50		It contains the localization of conserved and hydrophillic zone	[24]
30	Leu	Conserved residue in the IFNα types, IFNβ, and IFNω	
30	Leu	Bovine ifnar1/bovine ifnar2 produces an inactive complex with a ligand carrying incompatible substituent at that position	[38]
30	Leu	Site-direct mutagenesis showed that the substitution in that position is critical. Substitution in the indicated position reduced antiviral activity and antiproliferative activity of IFNs on human cells more than 100-fold	[24, 41, 89, 92]
30	Leu	The [Ala 30, 32, 33]IFNα2 analogue was an antagonist of the parent protein on bovine cells, but not on human cells	[39]
30	Leu	Single residue replacement in that position of IFNα2 produced an apparent drop in antiviral activity on human cells, in comparison with relatively stability of antiviral effect on bovine cells	[24]
30	Leu	Replacemement of Leu of human IFNα2 with Ile gave the relative activity on human cells of 30%	[93]
30	Leu	Replacemement of Leu of human IFNα2 with Asn gave the relative activity on human cells of 0.30%	[93]
30	Leu	Site-direct mutagenesis of Leu 30, Asp 32, and Arg 33 with Ala, Ala and Ala respectively produced inactive analogues on human cells in antiviral activity	[93]

Table 1. cont.

30	Leu	Residues Leu 30, Arg 33, and Phe 36 have been identified to be crucial for biological activity of IFNα4	[89]
30	Leu	The side chain of Leu 30 protrudes inward from the Loop AB region. The role of this residue appears to be a spacer between Loop AB and Helix E.	[42]
30	Leu	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	[37]
30–41		mAbs recognizing that structure inhibited the binding of IFNα2c on human and bovine cells, while mAbs to the corresponding region of IFNα1 lacked this ability	[94]
30–40		It seems the region where IFNα1 and IFNα2 have similar immunodominant epitopes	
30–35		Immunodominant epitope of neutralizing mAb in IFNα2c	[94, 95, 96]
30–38		Immunodominant epitope of neutralizing mAb in IFNα1	[94, 95, 96]
30–67		That fragment of IFNα2 and IFNα1 showed distincted antigenic properties by neutralization bioassay	[125]
31	Lys	Single replacement of Met 31 significantly contributed to low specific activity of IFNα1	[41]
31	Lys	Ligand lacking of a hydrophobic residue at the position 27 and having Met 31 instead of Lys (common situation in all the human and murine IFNs) produces an inactive complex with bovine ifnar1/murine ifnar2	[38]
31	Lys	Met 31 in human IFNα1 is not present in IFNs having optimal activity on human cells	[45]
31	Lys	It is proposed to be critical for the binding of B6. In this position IFNα2 is identical to IFNβ, but differs from IFNα1. In a 3D model of type I IFN there are three Lys 31, Lys 132, and Lys 134 spatially close each other. At pH 2 the binding of mAb B6 to IFNα2 is abrogated due to conformational change of this clusters	[77]
31	Lys	Site-direct mutagenesis of Lys 31 of human IFNα4 with Glu produced an analogue with antiviral activity on human, and bovine cells of 16%, and 72% respectively	[92]
31	Lys	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
31	Lys	Lys 31 has predicted to be contact residue at the interface region of the IFNα8 and the IFNα receptor subunit p40	
31	Lys	The [Glu 5, Ser 27, Met 31, Leu 60] IFNα2 analogue containing four residues of IFNα1, which do not occur in those positions in any other IFNα subtypes, had antiviral potency close to that of IFNα1	[41]
31	Lys	Endoproteinase Lys-C showed a rapid cleavage at that site	[27]
31	Lys	Site-direct mutagenesis of Lys 31 and Arg 33 of human IFNα4 with Glu and Glu respectively produced an analogue with antiviral activity on human and bovine cells of <0.003%, and <0.001%.	[92]
31	Lys	According to the Mu-IFNβ structure the side chain of Thr 31 on Loop AB is exposed	[42]
31–38		That segment in IFNα2 greatly influences that antiviral activity	[24]
31–51		Highly hydrophillic segment of human type I IFNs	[97]
32	Asp	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
32	Asp	Bovine ifnar1/bovine ifnar2 produces an inactive complex with a ligand carrying incompatible substituent at that position	[38]
32	Asp	Substitution of that conserved residue only slightly decreased the activity of IFNα4	[24]
32	Asp	Replacemement of Asp of human IFNα2 with: • Ala gave the relative activity on human cells of 200%. • Asn gave the relative activity on human cells of 40%.	[93]
32	Asp	The [Ala 30, 32, 33]IFNα2 analogue was an antagonist of the parent protein on bovine cells, but not on human cells	[39]
32	Asp	Site-direct mutagenesis of Leu 30 and Asp 32, and Arg 33 with Ala, Ala and Ala respectively produced inactive analogues on human bovine and murine cells in antiviral activity	[93]
33	Arg	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	

Table 1. cont.

33	Arg	Replacemement of Arg of human IFNα2 with: • Ala gave the relative activity on human cells of 0.04% • Lys gave the relative activity on human cells of 0.2% • Met gave the relative activity on human cells of <0.001%	[93]
33	Arg	Site-direct mutagenesis of Leu 30, Asp 32, and Arg 33 with Ala, Ala and Ala respectively produced an inactive analogue on human cells in antiviral activity	[93]
33	Arg	Bovine ifnar1/bovine ifnar2 produces an inactive complex with a ligand carrying incompatible substituent at that position	[38]
33	Arg	Site-direct mutagenesis of Arg 33 of human IFNα4 with: • Lys produced an analogue with antiviral activity on human and bovine cells of <0.1%, 5%: • Glu produced an analogue with antiviral activity on human and bovine cells of <0.1%, and <0.05%	[92]
33	Arg	Site-direct mutagenesis of Lys 31 and Arg 33 of human IFNα4 with Glu and Glu respectively produced analogues with antiviral activity on human and bovine cells of <0.003%, and <0.001%.	[92]
33	Arg	Residues Leu 30, Arg 33, and Phe 36 have been identified to be crucial for biological activity of IFNα4	[89]
33	Arg	Arg 33 is particularly sensitive to substitution and is highly conserved in IFNα, ω, and τ	[98, 99]
33	Arg	IFNα2 and IFNα4 differ in the epitopic presentation of residues of Loop AB around that position	[76]
33	Arg	That residue is necessary for the activity of IFNs on human cells	[92]
33	Arg	Site-direct mutagenesis showed that the substitution in that position is critical, since reduced antiviral activity and antiproliferative activity of IFNs on human cells is more than 100-fold	[24, 41, 89, 92]
33	Arg	The [Ala 30, 32, 33]IFNα2 analogue was an antagonist of the parent protein on bovine cells, but not on human cells	[39]
33	Arg	Site-direct mutagenesis of Arg 33 of human IFNα2c with His produced an analogue with antiviral activity on human, bovine cells of 2400%, and 206%	[43]
33	Arg	Site-direct mutagenesis of Arg 22 and Arg 33 of human IFNα2c with Lys and His respectively produced an analogue with antiviral activity on human and bovine cells of 2000%, and 124%	[88]
33	Arg	It is supposed that Arg 33 is in direct contact with the IFN receptor molecule	[42]
33	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
33	Arg	The side-chain of Arg 33 in the IFNα8 model is completely exposed to the solvent and is buried in the complex IFNα8/p40 receptor	[37]
33	Arg	In Mu-IFNβ the equivalent position is most probably Thr 31 and its side chain on Loop AB appears to be completely exposed	[100]
34	His	Single replacements in that position had only minor or insignificant effects on antiviral activity of IFNα4	[24]
34	His	The substitution in IFNα2c resulted in the local modification of the antigenic structure, but did not influence the antigenic properties of the rest of the molecule	[101]
34	His	The substitution in IFNα2c decreased the specific activity in human cell. The residues at the positions 23 and 34 are predicted to be exposed and sterically accessible	[43, 88]

Table 1. cont.

34	His	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
34–39		That fragment, corresponding to 32–47 of IFNβ, is the epitope of neutralizing mAbs, named mAb-A1 and mAb-A5	[102]
35	Asp	Single replacements in that position had only minor or insignificant effects on antiviral activity of IFNα4	[24]
36	Phe	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
36	Phe	Single residue replacement in that position of IFNα2 produced an apparent drop in antiviral activity on human cell, in comparison with relative stability of antiviral effect on bovine cells	[24]
36	Phe	In the sequence 29–36, Phe 36 is the only conserved residue in human IFNα2, α4,α1,α8, IFNω, murine IFNα, murine IFNβ	[38]
36	Phe	Site-direct mutagenesis showed that the substitution in that position is critical. Substitution in the indicated position reduced antiviral activity and antiproliferative activity of IFNs on human cells more than 100-fold	[24, 41, 89, 92]
36	Phe	Residues Leu 30, Arg 33, and Phe 36 have been identified to be crucial for biological activity of IFNα4	[89]
36	Phe	The integrity of the chain encompassing that conserved residue appears to be extremely critical for the binding and bioactivity on human cells, but just critical on bovine cells	[39]
36	Phe	In homology to Mu-IFNβ model, the side chains of Phe 36, Leu 67, Phe 68, Trp 141, and Val 143 form a hydrophobic core surrounding the side chain of Leu 131	[42]
36–41		Immunodominant epitope of neutralizing mAb is located in that region, which was shown to be most probably involved in the binding to cellular receptor	[94]
37	Gly	Single replacements in that position had only minor or insignificant effects on antiviral activity of IFNα4	[24]
37	Gly	Replacement of all five Gly residues of IFNα2 with chiral residues like Ala residues produced analogue [Ala 10, 37, 45, 103, 105] IFNα2, which is indistiguishable from IFNα2	[41]
37–50		That fragment of IFNα4 is recognized by antisera	[75, 103]
38	Phe	This positon affects the NK-2 reactivity	[24]
38	Phe	Among residues in variable position it is one of the most critical for antiviral activity of IFNα2	[24]
39	Pro	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
39	Pro	The equivalent position of IFNβ was critical for eliciting the biological activity	[104]
40	Gln	A considerable reduction in antiviral activity was found after substitution of non-conserved residues in IFNα4 and IFNα2	[24]
40	Gln	Substitution of Glu of IFNβ in the aligned sequence led to very large drop in bioactivity	[25, 106]
40–56		Fragment of IFNβ recognized by antisera	[102]
41	Glu	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
41	Glu	Site-direct mutagenesis of Glu 41 and Glu 42 of human IFNα4 with Lys and Lys respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 47%, (N.D.), and 92%	[92]
41	Glu	Substitution of that conserved residue only slightly decreased the activity of IFNα4	[104]
41–43		That fragment was important for the binding of neutralizing antibodies in IFNβ, in particular the conserved Pro 39 and Glu 41	[102]
42	Glu	Site-direct mutagenesis of Glu 41 and Glu 42 of human IFNα4 with Lys and Lys respectively produced an analogue with antiviral activity on human and bovine cells of 47%, and 92%	[92]
42	Glu	A considerable reduction in antiviral activity was found after substitution of non-conserved residues in IFNα4 and IFNα2	[24]
42–44		In human IFNα7c are significant for binding with receptor on bovine and human cell	[40]

Table 1. cont.

42–49		That fragment, corresponding to 40–47 of IFNβ, is the epitope of neutralizing mAbs, named mAb-A1 and mAb-A5	[102]
42–58		That fragment, corresponding to 40–56 of IFNβ, is the epitope of neutralizing mAbs, named mAb-A1 and mAb-A5	[102]
43	Phe	Conserved residue in the IFNα subtypes	
43	Phe	That residue of IFNα7c was significant for binding to receptor on bovine and human cells	[40]
44		Insertion of Asp 44 in IFNα2 does not modify the biological activity	[41]
44–45		The GG motif of bovine IFNαs at those positions seems interesting; the human IFNαs have DG, or GN in the case of IFNα2, which has a deletion at 44. IFNω and τ have EG and KG.The GG motif is present in bovine, feline, and porcine IFNαs. It is possible that the human receptor cannot cope with the degree of freedom available to a ligand having two consecutive residues without chirality in a sensitive region	[38]
45	Gly	Single replacement in that position had only minor or insignificant effects on antiviral activity of IFNα2	[89]
45	Gly	Replacement of all five Gly residues of IFNα2 with chiral residues like Ala residues produced analogue [Ala 10, 37, 45, 103, 105] IFNα2, which is indistiguishable from IFNα2	[41]
46	Asn	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
47	Gln	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
48	Phe	Conserved residue in the IFNα subtypes	
48	Phe	Single replacement in that position had only minor or insignificant effects on antiviral activity of IFNα2	[89]
48	Phe	Replacemement of Phe of human IFNα2 with: • Ser gave the relative antiviral activity on human, murine and bovine cell of 190%, 190%, and 40%. • Tyr gave the relative antiviral activity on human, murine and bovine cell of 140%, 140%, and 160% Cys gave the relative antiviral activity on human, murine and bovine cell of 110%, 110%, and 420%	[105]
49	Gln	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
49	Gln	Replacemement of Gln of human IFNα2 with His gave the relative antiviral activity on human, murine and bovine cells of 120%, 120%, and 80% respectively.	[105]
49	Gln	It was without effect on the antiviral and antiproliferative activity of IFNα2	[24]
50	Lys	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
50	Lys	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
54	Ile	Conserved residue in the IFNα subtypes	
58	His	Conserved residue in the IFNα subtypes and IFNω	
59	Glu	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
60	Met	The [Glu 5, Ser 27, Met 31, Leu 60] IFNα2 analogue containing four residues of IFNα1, which do not occur in those positions in any other IFNα subtypes, had antiviral potency close to that of IFNα1	[41]
60	Met	Single mutation of each of Met 16, 21, 60, 112, and 149 of IFNα2 with Leu residues, and contemporary substitution of all Met residues with Leu residues produced analogues indistiguishable from unmodified IFNα2	[41]
62	Gln	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	

Table 1. cont.

62	Gln	When the residue Glu, conserved in all the human, murine, and bovine IFNαs and human IFNβ is replaced by Lys antiviral activity remaned unchanged	[105]
62–122		This region of IFNα 2 is assumed to bind the bovine receptor	[108]
63	Gln	Conserved residue in the IFNα subtypes and IFNω	
63–67		mAbs recognizing that region of IFNα1 inhibited the binding to bovine and human cells	[24]
63–85		Second immunodominant epitope for IFNα1	[94, 96]
63–86		In IFNα2 this region is not silent from the view of activity on human cells. The hybrid IFNα2/α1/α2 with only three residues specific for IFNα1 in positions 69, 80, and 86 had 7-times decreased specific activity on human cells as compared to IFNα2	[107]
63–78		Epitope of mAb neutralizing the antiviral and antiproliferative activity of IFNα2	[107]
65	Phe	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
66	Asn	Conserved residue in the IFNα subtypes	
67	Leu	In homology to Mu-IFNβ model , the side chains of Phe 36, Leu 67, Phe 68, Trp 141, and Val 143 form a hydrophobic core surrounding the side chain of Leu 131	[42]
68	Phe	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
68	Phe	In homology to Mu-IFNβ model , the side chains of Phe 36, Leu 67, Phe 68, Trp 141, and Val 143 form a hydrophobic core surrounding the side chain of Leu 131	[42]
69	Ser	Hybrid IFNα2/α1/α2 with only three residues specific for IFNα1 in positions 69, 80, and 86 had 7-times decreased specific activity on human cells as compared to IFNα2	[109]
69	Ser	Replacement of Ser 69 of IFNα2 by Thr from IFNα1 resulted in a relatively greater decrease in NK-boosting potency than in antiviral or antiproliferative activity	[1]
69–79		Highly hydrophillic segment of human type I IFNs	[97]
69–80		The corresponding synthetic fragment showed weak antiproliferative activity, but potentiated the biological activity of IFNα1 and IFNα2	[109]
70	Thr	Conserved residue in the IFNα subtypes and IFNω	
73	Ser	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
74	Ser	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
74	Ser	Mutation in that position of IFNα2 resulted in a loss of antiviral activity on human cells	[24]
77	Trp	Chemical modification of Trps of IFNβ equivalent to the positions 77 and 141 of the IFNα2 aligned sequence causes dramatic loss of antiviral activity.	[104]
77–85		Epitope of mAb neutralizing the antiviral and antiproliferative activity of IFNα2	[107]
78	Asp	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
78–95		Differential sensitivites between IFNαs and IFNβ may be associated with that region, specifically at residues 84 (Lys in IFNα2, aligned position in IFNβ Asn), 86 (Tyr in IFNα2, aligned position in IFNβ Leu), 87 (Thr in IFNα2, alligned position in IFNβ Ala)	[25]
79	Glu	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
80	Thr	Hybrid IFNα2/α1/α2 with only three residues specific for IFNα1 in positions 69, 80, and 86 had 7-times decreased specific activity on human cells as compared to IFNα2	[107]
81	Leu	Conserved residue in the IFNα subtypes and IFNω	
82	Leu	Conserved residue in the IFNα subtypes and IFNω	
83	Asp	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
84	Lys	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]

Table 1. cont.

84	Lys	IFNα8 contains acidic residues at positions 84 and 90, while other IFNα subtypes do not. Those residues are responsible for the anomalous migration in SDS-PAGE	[110]
84	Lys	The doubled mutation Lys 84 to Glu and Tyr 90 to Asp abolished the activity of the hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] on ordinary mouse cells but not on mouse cell expressing human ifnar1	[91]
84	Lys	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] showed partial activity on murine cells. Point mutations that change the individual of those of IFNα8 (i.e. Lys 84 to Glu, Cys 86 to Tyr, Thr 87 to Ile and Tyr 90 to Asp) produced marked losses of activity on mouse cells	[91, 111]
84	Lys	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with Lys 84 and a point mutation Tyr 90 to Asp turned out to be the fastest IFNα in the dynamics of the complex formation with IFNα receptor	[48]
84	Lys	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with Tyr 90-Lys 84, which are the couple of human IFNα2, was slower in the dynamics of the complex formation with IFNα receptor	[48]
84	Lys	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with Asp 90-Glu 84, which are the couple of human IFNα8, was slower in the dynamics of the complex formation with IFNα receptor	[48]
84	Lys	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with a point mutation Tyr 90- Glu 84 was very slow in the dynamics of the complex formation with IFNα receptor	[48]
84	Lys	Different sensitivities between IFNαs and IFNβ may be associated to the residues 84 (Lys in IFNα2, Asn in the aligned position of IFNβ), 86 (Tyr in IFNα2, Leu in IFNβ), 87 (Thr in IFNα2,Ala in IFNβ Ala)	[25]
86	Tyr	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] showed partial activity on murine cells. Point mutations that change the individual of those of IFNα8 (i.e. Lys 84 to Glu, Cys 86 to Tyr, Thr 87 to Ile and Tyr 90 to Asp) produced marked losses of activity on mouse cells	[91, 111]
86	Tyr	Hybrid IFNα2/α1/α2 with only three residues specific for IFNα1 in positions 69, 80, and 86 had 7-times decreased specific activity on human cells as compared to IFNα2	[107]
86	Tyr	Site-direct mutagenesis of Ser 86 of human IFNα4 with Cys from IFNα1 sequence produced analogue with antiviral activity on human, murine, and bovine cells of 91%, 1367%, and 125%	[44]
86	Tyr	Site-direct mutagenesis of Ser 86 and Arg 121 of human IFNα4 with Cys and Lys from IFNα1 sequence produced analogue with antiviral activity on human, murine, and bovine cells of 395%, 4667%, and 116%	[44]
86	Tyr	Different sensitivities between IFNαs and IFNβ may be associated with the residues 84 (Lys in IFNα2, Asn in the alligned position of IFNβ), 86 (Tyr in IFNα2, Leu in IFNβ), 87 (Thr in IFNα2,Ala in IFNβ Ala)	[25]
86	Tyr	The anologue of IFNα1 with the substitution of Cys 1, 86, and 99 with Ser residues retained 23% of biological activity in human cells, but the activity was not affected on bovine cells	[119]
87	Thr	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] showed partial activity on murine cells. Point mutations that change the individual of those of IFN(8 (i.e. Lys 84 to Glu, Cys 86 to Tyr, Thr 87 to Ile and Tyr 90 to Asp) produced marked losses of activity on mouse cells	[91, 111]
87	Thr	Different sensitivities between IFNαs and IFNβ may be associated to the residues 84 (Lys in IFNα2, Asn in the aligned position of IFNβ), 86 (Tyr in IFNα2, Leu in IFNβ), 87 (Thr in IFNα2,Ala in IFNβ Ala)	[25]
88	Glu	Conserved residue in the IFNα subtypes and IFNω	
88	Glu	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]

Table 1. cont.

90	Tyr	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] showed partial activity on murine cells. Point mutations that change the individual of those of IFNα8 (i.e. Lys 84 to Glu, Cys 86 to Tyr, Thr 87 to Ile and Tyr 90 to Asp) produced marked losses of activity on mouse cells	[91, 111]
90	Tyr	The doubled mutation Lys 84 to Glu and Tyr 90 to Asp abolished the activity of hybrid IFN[α8(1–60), α1(61–92), α 8(93–166)] on ordinary mouse cells but not on mouse cell expressing human ifnar1	[91]
90	Tyr	IFNα8 contains acidic residues at positions 84 and 90, while other IFNα subtypes do not. Those residues are responsible for the anomalous migration in SDS-PAGE	[110]
90	Tyr	Hybrid IFN[α8(1–60), α1(61–92), α 8(93–166)] with a point mutation Tyr 90 to Asp and with the couple Asp 90/Lys 84 turned out to be the fastest IFNα in the dynamics of the complex formation with IFNα receptor	[48]
90	Tyr	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with Tyr 90-Lys 84, which are the couple of human IFNα2, was slower in the dynamics of the complex formation with IFNα receptor	[48]
90	Tyr	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with Asp 90-Glu 84 was slower in the dynamics of the complex formation with IFNα receptor	[48]
90	Tyr	Hybrid IFN[α8(1–60), α1(61–92), α8(93–166)] with Tyr 90-Glu 84, which are the couple of human IFNα8, is very slow in the dynamics of the complex formation with IFNα receptor	[48]
90	Tyr	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
91	Gln	Conserved residue in the IFNα subtypes and IFNω	
92	Gln	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
94	Asn	Conserved residue in the IFNα subtypes and IFNβ	
95	Asp	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
97	Glu	Conserved residue in the IFNα subtypes and IFNβ	
97	Glu	Negatively charged residue in that position (Asp 78, Glu 79, Asp 83, Glu 84, Glu 88, Asp 90, Asp 95, and Glu 97) has been predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
98–114		Substitution of that segment of IFNα2 with that completely unrelated of IFNγ did not have effect on the antiviral activity	[41]
99	Cys	The anologue of IFNα1 with the substitution of Cys 1, 86, and 99 with Ser residues retained 23% of biological activity in human cells, but the activity was not affected on bovine cells	[119]
100	Val	The hybrid [Met 100, Glu 102, Glu 103, Arg 104, Gly 106, Asn 112, Ala 113] IFNα2 analogue showed biological activity indistinguishable from the parent protein	[41]
102	Gln	Conserved residue in the IFNα subtypes and IFNω	
102–110		This segment could be deleted in IFNα2 without abolishing the bioactivity	[114]
102–111		Deleted IFNα2 analogue maintained antiviral activity with only little effect on mAb-NK-2 reactivity	[41]
103	Gly	Replacement of all five Gly residues of IFNα2 with chiral residues like Ala residues produced analogue [Ala 10, 37, 45, 103, 105] IFNα2, which is indistiguishable from IFNα2	[41]
105	Gly	Replacement of all five Gly residues of IFNα2 with chiral residues like Ala residues produced analogue [Ala 10, 37, 45, 103, 105] IFNα2, which is indistiguishable from IFNα2	[41]

Table 1. cont.

106	Thr	Natural IFNα2 resulted to be glycosilated at that position	[112]
111	Leu	Conserved residue in the IFNα subtypes	
112	Met	Conserved residue in the IFNα subtypes	
112	Met	Single mutation of each of Met 16, 21, 60, 112, and 149 of IFNα2 with Leu residues, and contemporary substitution of all Met residues with Leu residues produced analogues indistiguishable from unmodified IFNα2	[41]
112–121		The epitope of mAb-NK-2 is within that fragment	[113]
112–148		Immunodominant epitope of neutralizing mAb is located in that region, which could be responsible for inducing antiviral and antiproliferative activity	[94]
113	Lys	Substitution of Lys 113 and Glu 114 of IFNα2 with Asn and Ala from IFNα1 forbids the reaction with mAb-NK-2	[41]
113	Lys	Analogue of IFNα21 where Asn 113, Val 114, and Lys 121 were replaced by those of IFNα2, Lys 113, Gly 114, and Ala 121, was bound by mAb-NK-2, while IFNα21 did not	[113]
113–139		mAb, designed 4F2, raised against IFNαcons, recognized that region and neutralized the antiviral activity of IFNαcons, IFNα2, and IFNα1	[82]
114	Glu	The subvariant of IFNα4 are antigenically distinct in this epitope because of the substitution Glu(4a) to Val(4b)	[24]
114	Glu	Critical residue of IFNα2 for the epitope of mAb, named LI-1, are localized at position 114 and 121. MAb-LI-1 neutralize also IFNα2, IFNα10, IFNα6, IFNα7, but not IFNα1D and IFNα21. IFNα1D and IFNα21 have at position 114 and 121 Val and Lys, respectively	[114]
114	Glu	Substitution of Lys 113 and Glu 114 of IFNα2 with Asn and Ala from IFNα1 forbids the reaction with mAb-NK-2	[41,113]
114	Glu	Analogue of IFNα21 where Asn 113, Val 114, and Lys 121 were replaced by those of IFNα2, Lys 113, Gly 114, and Ala 121, was bound by mAb-NK-2, while IFNα21 did not	[113]
115	Asp	Conserved residue in the IFNα subtypes	
117	Ile	Conserved residue in the IFNα subtypes	
118	Leu	Conserved residue in the IFNα subtypes, IFNβ, and IFNω	
118–131		Simultaneous substitutions of the positions 118, 123, 126, 127, 130, 131 of IFNα2 and of IFN β by Alas produced an inactive analogue	[41]
119	Ala	For NK-2 epitope this position is critical	[41]
120	Val	Conserved residue in the IFNα subtypes	
120–145		The segment is comprised of two clusters of conserved hydrophillic residues. Both convergent helices stabilize the conformation of the hydrophilic DE loop	[115, 116]
120–145		It contains the localization of the most conserved hydrophillic zone	[24]
121	Arg	For NK-2 epitope this position is critical	[41, 113]
121	Arg	Site-direct mutagenesis of Arg 121 and Lys 122 of human IFNα4 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 8% and 29%	[92]
121	Arg	Site-direct mutagenesis of Arg 121and Lys 122 of human IFNα4 with Glu and Glu respectively produced an analogue with antiviral activity on human and bovine cells of 0.2% and 0.054%	[44]
121	Arg	Site-direct mutagenesis of Ser 86 and Arg 121 of human IFNα4 with Cys and Lys of IFNα1 respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 395%, 4667%, and 116%	[44]
121	Arg	Site-direct mutagenesis of Arg 121, Lys 122, and Tyr 123 of human IFNα4 with Glu, Glu, and Ala respectively produced an analogue with antiviral activity on human and bovine cells of 0.059% and 0.021%	[44]
121	Arg	Analogue of IFNα21 where Asn 113, Val 114, and Lys 121 were replaced by those of IFNα2, Lys 113, Gly 114, and Ala 121, was bound by mAb-NK-2, while the parent protein was not	[113]

Table 1. cont.

121	Arg	Critical residues of IFNα2 for the epitope of mAb, named LI-1, are localized at position 114 and 121. MAb-LI-1 neutralize also IFNα10, IFNα6, IFNα7, but not IFNα1D and IFNα21. IFNα1D and IFNα21 have at position 114 and 121 Val and Lys, respectively	[114]
121	Arg	Analogue of IFNα21 where Asn 113, Val 114, and Lys 121 were replaced by those of IFNα2, Lys 113, Gly 114, and Ala 121, was bound by mAb-NK-2, while IFNα21 did not	[113]
121	Arg	Site-direct mutagenesis of Arg 121 of human IFNα4 with Lys produced an analogue with antiviral activity on human, murine, and bovine cells of 240%, 2034%, and 115%	[44]
121	Arg	Site-direct mutagenesis of Ser 86 and Arg 121 of human IFNα4 with Cys and Lys respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 395%, 4667%, and 116%	[44]
121	Arg	Point mutation at that position alters the activity of IFNα2 on murine cells	[45]
121	Arg	Site-direct mutagenesis of Arg 121 of human IFNα2 with Lys produced an analogue with antiviral activity on human, murine, and bovine cells of 120%, 1700%, and 90%	[45]
121	Arg	Site-direct mutagenesis of Arg 121, Gln 125 of human IFNα2 with Lys and Arg respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 130%, 40400%, and 150%	[45]
121	Arg	Site-direct mutagenesis of Arg 121, Gln 125, and Lys 132 of human IFNα2 with Lys, Arg and Thr respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 150%, 16900%, and 130%	[45]
121	Arg	The positions 121, 125, 128, and 132 on the D helix are exposed to the solvent and are symmetric about a straight line with angular deviations of 10 and 30°	[27, 42]
121	Arg	Site-direct mutagenesis of Lys 121 and Lys 122 of human IFNα1 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 3% and 75%	[46]
121	Arg	Site-direct mutagenesis of Lys 121 and Lys 122 of human IFNα1 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 3% and 75%	[92]
121	Arg	Residue on Helix D that is equally important for both human and murine receptor interaction	[42]
121–123		Large changes of activity of humans IFNαs are due to that fragment	[44, 92]
121–145		Highly hydrophillic segment of human type I IFNs	[97]
122	Lys	Conserved residue in the IFNα subtypes	
122	Lys	Site-direct mutagenesis of Arg 121 and Lys 122 of human IFNα4 with Glu and Glu respectively produced an analogue with antiviral activity on human and bovine cells of 0.2% and 0.054%	[44]
122	Lys	Site-direct mutagenesis of Lys 121 and Lys 122 of human IFNα1 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 3% and 75%	[46]
122	Lys	Site-direct mutagenesis of Arg 121 and Lys 122 of human IFNα4 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 8% and 29%	[92]
122	Lys	Site-direct mutagenesis of Arg 121, Lys 122, and Tyr 123 of human IFNα4 with Glu, Glu, and Ala respectively produced an analogue with antiviral activity on human and bovine cells of 0.059% and 0.021%	[44]
123	Tyr	Site-direct mutagenesis of Tyr 123 of human IFNα1 with Gly produced an analogue with antiviral activity on human, murine, and bovine cells of <0.2%, 110%, and 62%	[46]

Table 1. cont.

123	Tyr	Replacemement of Tyr of human IFNα1 with: • Phe gave the relative activity on human, murine and bovine cells of 62%, 48%, and 104% • Trp gave the relative activity on human, murine and bovine cells of 38%, 18%, and 129% • Lys gave the relative activity on human, murine and bovine cells of 2.5%, 5%, and 95% • Asp gave the relative activity on human, murine and bovine cells of 3.5%, 24%, and 36% • Ser gave the relative activity on human, murine and bovine cells of 11%, 2.5%, and 141% • Ala gave the relative activity on human, murine and bovine cells of 8.3%, 3.4%, and 172%	[47]
123	Tyr	Deletion of Tyr of human IFNα1 gave the relative activity on human, murine and bovine cell of 1.2%, <0.8% and 1.4%	[47]
123	Tyr	Site-direct mutagenesis of Tyr 123 of human IFNα4 with Ser produced an analogue with antiviral activity on human, and bovine cells of 8% and 30%	[92]
123	Tyr	Site-direct mutagenesis of Tyr 123 of human IFNα1 with Ser produced an analogue with antiviral activity on human, and bovine cells of 11% and 141%	[92]
123	Tyr	Permissive change at 123 and its position suggest an importance in maintaining the conformation of the D helix relative to the rest of the molecule	[42, 100]
123	Tyr	Site-direct mutagenesis of Arg 121, Lys 122, and Tyr 123 of human IFNα4 with Glu, Glu, and Ala respectively produced an analogue with antiviral activity on human and bovine cells of 0.059% and 0.021%	[44]
123	Tyr	Deletion (but not substitution even by small hydrophillic residues) causes loss of both activity and binding of human IFNα1 on bovine cells. If the helix is maintained, the deletion changes the identity of the position 125, 128, 132	[47]
123	Tyr	In the Mu-IFNβ model the side chain of Tyr corresponding at that position on Helix D is oriented towards the inside of the molecule. This residue appears to play a role of spacer between Helix D abd Loop AB	[42]
123	Tyr	Residue on Helix D that is equally important for both human and murine receptor interaction	[42]
123	Tyr	In the proposed model the following positions of IFNαcons are spatially accessible: 123, 125, 126, 128–130, 132–139	[25]
123	Tyr	A modification at Tyr 123 leads to an order of magnitude or more loss of activity in human and murine IFNs, but not bovine	[47, 92]
123	Tyr	Replacement with Gly produced a loss of 99% of antiviral acivity on human cells	[119]
123–131		It forms the hydrophilic surface of the D helix	[115, 116]
123–140		The domain is in close spatial proximity to the fragment 29–35 in the proposed model of IFNαcons, where the positions 123, 125, 126, 128–130, 132–139 are spatially accessible	[25]
124	Phe	Conserved residue in the IFNα subtypes and IFNω	
124	Phe	Site-direct mutagenesis of Phe 124 of human IFNα1 with Ser produced an analogue with antiviral activity on human, murine, and bovine cells of 75%, 200%, and 207%	[46]
124	Phe	Site-direct mutagenesis of Phe 124 of human IFNα1 with Ser produced an analogue with antiviral activity on human and bovine cells of 62% and 160%	[92]
124–138		Antisera against that synthetic peptide corresponding to that fragment of IFNα2 neutralized the activity	[24]
124–138		Synthetic C-fragment of IFNα2 possessed IFN-like antiviral activity and was able to bind to human blood leukocytes	[120, 117]
125	Gln	Site-direct mutagenesis of Arg 125 of human IFNα1 with Gln produced an analogue with antiviral activity on human, murine, and bovine cells of 27%, 0.7%, and 72%	[46]
125	Gln	Site-direct mutagenesis of Arg 125 of human IFNα1 with Gln produced an analogue with antiviral activity on human, and bovine cells of 26% and 140%	[92]
125	Gln	Point mutation at that position alter the activity of IFNα2 on murine cells	[45]

Table 1. cont.

125	Gln	The positions 121, 125, 128, and 132 on the D helix are exposed to the solvent and are symmetric about a straight line with angular deviations of 10 and 30°	[27, 42]
125	Gln	Site-direct mutagenesis of Gln 125 of human IFNα2 with Arg from IFNα1 sequence produced an analogue with antiviral activity on human, murine, and bovine cells of 110%, 14200%, and 180%	[45]
125	Gln	Site-direct mutagenesis of Gln 125 of human IFNα2 with His produced an analogue with antiviral activity on human, murine, and bovine cells of 110%, 920%, and 230%	[45]
125	Gln	Site-direct mutagenesis of Gln 125 of human IFNα2 with Leu produced an analogue with antiviral activity on human, murine, and bovine cells of 110%, 500%, and 140%	[45]
125	Gln	Site-direct mutagenesis of Arg 121, Gln 125, and Lys 132 of human IFNα2 with Lys, Arg and Thr from IFNα1 sequence produced an analogue with antiviral activity on human, murine, and bovine cells of 150%, 16900%, and 130%	[45]
125	Gln	Site-direct mutagenesis of Arg 121, Gln 125 of human IFNα2 with Lys and Arg from IFNα1 sequence produced an analogue with antiviral activity on human, murine, and bovine cells of 130%, 40400%, and 150%	[45]
125	Gln	Site-direct mutagenesis of Gln 125 and Lys 132 of human IFNα2 with Arg and Thr from IFNα1 sequence produced an analogue with antiviral activity on human, murine, and bovine cells of 155%, 2300%, and 160%	[45]
125	Gln	The residue on Helix D appear to be important for murine but not for human target cells	[42]
125	Gln	In the proposed model the following positions of IFNαcons are spatially accessible: 123, 125, 126, 128–130, 132–139	[25]
126	Arg	Conserved residue in the IFNα subtypes and IFNβ	
126	Arg	In the proposed model the following positions of IFNαcons are spatially accessible: 123, 125, 126, 128–130, 132–139	[25]
127	Ile	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
128	Thr	Conserved residue in the IFNα subtypes	
128	Thr	The positions 121, 125, 128, and 132 on the D helix are exposed to the solvent and are symmetric about a straight line with angular deviations of 10 and 30°	[27, 42]
129	Leu	Conserved residue in the IFNα subtypes	
129–144		Antisera against the synthetic peptide corresponding to that fragment of IFNα2 neutralized the activity	[24]
130	Tyr	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
130	Tyr	Site-direct mutagenesis of Tyr 130 of human IFNα4 with: • Phe produced an analogue with antiviral activity on human and bovine cells of 92% and 68% • Gly produced an analogue with antiviral activity on human and bovine cells of 2% and 9% • Arg produced an analogue with antiviral activity on human and bovine cells of 16% and 26%	[44]
130	Tyr	In the Mu-IFNβ model the side chain of Tyr corresponding at that position protrudes from Helix D towards the Loop AB. This residue appears to play a role of spacer between Helix D and Loop AB	
131	Leu	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
131	Leu	Substitution of Leu 131 diminuished the antiproliferative activity of IFNα4	[92]
131	Leu	Site-direct mutagenesis of Leu 131 of human IFNα4 with Pro produced an analogue with antiviral activity on human cells of <0.2%	[44]
131	Leu	Site-direct mutagenesis of Leu 131 and Thr 132 of human IFNα4 with Gln and Lys respectively produced an analogue with antiviral activity on human and bovine cells of 77% and 53%	[44]

Table 1. cont.

131	Leu	Point mutation at that position alter the activity of IFNα2 on murine cells	[45]
131	Leu	Leu 131 is perfectly conserved in all the human Type I IFNs. In the Mu-IFNβ model the side chain of Leu corresponding at that position on Helix D is completely buried and surrounded by a group of hydrophobic side chains from Phe 36, Leu 67, Phe 68, Trp 141, and Val 143 to form a small hydrophobic core	[42]
131–138		Synthetic fragment corresponding to IFNα2 sequence showed immunomodulatory activity without inducing antiviral protection	[122]
132	Lys	Site-direct mutagenesis of Lys 132 of human IFNα2 with Thr produced analogue with antiviral activity on human, murine, and bovine cells of 90%, 10%, and 110%	[45]
132	Lys	Site-direct mutagenesis of Gln 125 and Lys 132 of human IFNα2 with Arg and Thr respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 155%, 2300%, and 160%	[45]
132	Lys	Site-direct mutagenesis of Arg 121, Gln 125, and Lys 132 of human IFNα2 with Lys, Arg and Thr respectively produced an analogue with antiviral activity on human, murine, and bovine cells of 150%, 16900%, and 130%	[45]
132	Lys	It is proposed to be critical for the binding of mAb B6. In this position IFNα2 is identical to IFNβ, but differs from IFNα1. In a 3D model of type I IFN there are three Lys 31, Lys 132, and Lys 134 spatially close to each other. At pH 2 the binding of mAb B6 to IFNα2 is abrogated due to conformational change of this clusters	[77]
132	Lys	It is proposed to be critical for the binding of mAb B6. (See also 31)	[77]
132	Lys	Replacement in that variable position had relatively small effect on antiviral potency of IFNα4 on human and bovine cells	[44, 92]
132	Lys	The residue on Helix D appear to be important for murine but not for human target cells	[42]
132	Lys	The positions 121, 125, 128, and 132 on the D helix are exposed to the solvent and are symmetric about a straight line with angular deviations of 10 and 30°	[27, 42]
132	Lys	Variant IFNα7c, which differs from IFNα7a for Thr 132 instead of Met, was neutralized by a mixture of mAbs [118], while IFNα did not	[74]
132–137		The sequence of IFNα2 is identical to IFNω	[24, 126]
132–137		It represents the only evidence of an antigenic homology between human IFNα and IFNβ. This common epitope recognized by mAb named B6 is conformational. B6 did not neutralize IFNα1 even when its sequential homology with IFNα2 is higher than that of corresponding parts between IFNα2 and IFNβ	[77]
132–138		It is supposed to be important for the low-affinity binding	[115]
132–138		The shortest interhelical segment. It corresponds to the region with maximal hydrophilicity in IFNα2 and IFNω, but it was not reflected in immunodominant properties of this structure	[24]
132–139		In the proposed model the following positions of IFNαcons are spatially accessible: 123, 125, 126, 128–130, 132–139	[25]
133	Glu	Replacement in that variable position had relatively small effect on antiviral potency of IFNα4 on human and bovine cells	[24]
133	Glu	Site-direct mutagenesis of Glu 133 of human IFNα4 with Lys produced an analogue with antiviral activity on human, murine, and bovine cells of 143%, 1502, and 90%	[44]
133	Glu	The residue on LoopDE appears to be important for murine but not for human target cells	[44]
133–142		mAb raised to synthetic peptide corresponding to that fragment neutralized antiviral effect of leukocyte IFNα (purified by NK-2 antibody) without affecting its antiproliferative activity	[80]
133–147		mAb to that fragment of IFNα2 neutralized the biological activity	[80]
134	Lys	Site-direct mutagenesis of Lys 134 and Lys 135 of human IFNα1 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 33% and 93%	[92]

Table 1. cont.

134	Lys	Site-direct mutagenesis of Lys 134 and Lys 135 of human IFNα4 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 31% and 70%	[92]
134	Lys	It is proposed to be critical for the binding of B6. In this position IFNα2 is identical to IFNβ. In a 3D model of type I IFN there are three Lys 31, Lys 132, and Lys 134 spatially close each other. At pH 2 the binding of mAb B6 to IFNα2 is abrogated due to conformational change of this clusters	[77]
135	Lys	Conserved residue in the IFNα subtypes and IFNω	
135	Lys	Site-direct mutagenesis of Lys 134 and Lys 135 of human IFNα4 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 31% and 70%	[92]
135	Lys	Site-direct mutagenesis of Lys 134 and Lys 135 of human IFNα1 with Leu and Leu respectively produced an analogue with antiviral activity on human and bovine cells of 33% and 93%	[92]
135	Lys	Replacement in that variable position had relatively small effect on antiviral potency of IFNα4 on human and bovine cells	[24]
136	Tyr	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
136	Tyr	Site-direct mutagenesis of Tyr 136 of human IFNα1 with Gly produced an analogue with antiviral activity on human and bovine cells of 102% and 145%	[92]
136	Tyr	Site-direct mutagenesis of Tyr 136 of human IFNα1 with His produced an analogue with antiviral activity on human, murine, and bovine cells of 80%, 500%, and 103%	[46]
136	Tyr	The residue on Loop DE appear to be important for murine but not for human target cells	
136–138		Following binding activity of IFNα2 peptides to thymocytes, it has been recognized as critical for receptor interaction (Tyr-Ser-Pro)	[122]
137	Ser	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
137	Ser	Change in that position equivalent to 139 of IFNβ greatly reduced the biological activity	[104]
138	Pro	The side chains of Phe 36, Val 65, Phe 66, Trp 138, and Val 141 form a hydrophobic core surrounding the side chain of Leu 131	[42]
138	Pro	Change in that position equivalent to 140 of IFNβ greatly reduced the biological activity	[104]
138–166		Deleted segment in IFNα2 lacked completely antiviral activity	[41]
139	Cys	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
139	Cys	[Ser 29, Ser 139] IFNα2 showed reduced but still detectable antiviral activity	[41]
139	Cys	[Gly 29, Gly139] IFNα2 analogue was inactive	[41]
139	Cys	[Ala 139, Cys 140]-IFNα2, where the sequence of the position 139 and 140 are inverted, is devoid of biological activity on human cells	[41]
139–145		It forms a hydrophilic surface of the E helix	[115, 116]
139–149		A region highly susceptible to proteolytic cleavages	[24]
140	Ala	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
141	Trp	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
141	Trp	In homology to Mu-IFNβ model , the side chains of Phe 36, Leu 67, Phe 68, Trp 141, and Val 143 form a hydrophobic core surrounding the side chain of Leu 131	[42]
141	Trp	Chemical modification of Trps of IFNβ equivalent to the positions 77 and 141 of the IFNα2 alligned sequence causes dramatic loss of antiviral activity	104

Table 1. cont.

142	Glu	Conserved residue in the IFNα subtypes, and IFNω	
142–151		Policlonal antisera obtained by immunization with synthetic peptide corresponding to that region reacted with IFNα2a,b, IFNα4a, IFNα14, and IFNβ	[75, 103]
143	Val	Conserved residue in the IFNα subtypes, and IFNω	
143	Val	In homology to Mu-IFNβ model, the side chains of Phe 36, Leu 67, Phe 68, Trp 141, and Val 143 form a hydrophobic core surrounding the side chain of Leu 131	[42]
143–166		The analogue of IFNα2 with that deleted fragment lacked completely antiviral activity	[41]
144	Val	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
145	Arg	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
145	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
145	Arg	Change in that position equivalent to 147 of IFNβ greatly reduced the biological activity	[104]
146	Ala	Conserved residue in the IFNα subtypes	
146	Ala	Change in that position of IFNα2 diminuished the biological activity	[24]
147	Glu	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
147–166		The analogue of IFNα2 with that deleted fragment lacked completely antiviral activity	[41]
148	Ile	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
148	Ile	Change in that position equivalent to 150 of IFNβ greatly reduced the biological activity	[104]
149	Met	Conserved residue in the IFNα subtypes, and IFNω	
149	Met	Single mutation of each of Met 16, 21, 60, 112, and 149 of IFNα2 with Leu residues, and contemporary substitution of all Met residues with Leu residues produced analogues indistiguishable from unmodified IFNα2	[41]
149–166		The analogue of IFNα2 with that deleted fragment lacked completely antiviral activity	[41]
150	Arg	Conserved residue in the IFNα subtypes and IFNβ	
150	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
150	Ser	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	
151	Ser	Conserved residue in the IFNα subtypes, and IFNω	
151	Ser	Change in that position of IFNα2 diminuished the biological activity	[24]
151–166		mAb to this region did not prevent the binding of IFNα2 to the bovine receptor	[69, 124]
151–166		Truncation of that segment in IFNα2 analogue exerted minimal effect on bovine cells	[120]
151–166		The analogue of IFNα2 with that deleted fragment lacked completely antiviral activity	[120]
152	Phe	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	[37]

Table 1. cont.

152–166		Analogue of IFNα4 with that deleted segment had reduced antiviral activity to 0.2%	[44]
153	Ser	Conserved residue in the IFNα subtypes	
153	Ser	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]
154	Leu	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	
154–166		C-terminal 13 residues of IFNα2 removed by proteolytic cleavage had specific activity close to that of full-length protein	[121]
155	Ser	Conserved residue in the IFNα subtypes and IFNω	
155–166		C-terminal truncated recombinant IFNα2 variant exhibited biological activity 3–4 times lower than the complete molecule	[41]
156	Thr	A cluster of hydrophobic residues of IFNα8, Leu 9, Ile 16, Leu 17, Leu 30, Phe 152, Leu 154, Ile 156, are predicted to be involved in the binding to the receptor (site I)	[37]
156	Thr	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]
156–166		C-terminal truncated IFNα2 analogue exhibited biological activity 3–4 times lower than the complete molecule	[41]
157	Asn	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]
157–166		C-terminal truncated IFNα2 analogue obtained by proteolytic cleavage had specific activity close to that of full-lenght protein	[122]
158–166		Deletion of that fragment in IFNα4 reduced antiviral activity to 3%	[44]
160	Glu	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
160	Glu	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]
161	Ser	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]
161–166		C-terminal truncated recombinant IFNα2 variant exhibited biological activity 3–4 times lower than the complete molecule	[41]
162	Leu	Conserved residue in the IFNα subtypes, IFNβ and IFNω	
163	Arg	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
163	Arg	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]
163–172		Truncated IFNω was still biologically active	[123]
165	Lys	Conserved residue in the IFNα subtypes, and IFNω	
165	Lys	A cluster of positively charged residues, His 7, Arg 12, Arg 13, Arg 22, Arg 23, Lys 31, Arg 33, His 34, Lys 46, Lys 50, Arg 145, Arg 150, Lys 160, Lys 163, Lys 165 are predicted in IFNα8 to form a continuous surface of positive electrostatic potential complementary to negative potential of receptor molecule	[37]
165	Lys	In the proposed model the following residues of IFNαcons are spatially accessible: Arg 150, Ser 153, Thr 156, Asn 157, Glu 160, Arg 161, Arg 163, Arg 164, Lys 165, Glu 166	[25]

All these structural constraints are important to properly set-up the hot segments, namely 29–35 and 123–140, which must be closely located to make Type I IFNs biologically active [25].

The situation described above should be considered as a sort of "average" among Type I IFN moieties, while differences can be noticed when single IFNα subtypes, IFNβ, or IFNω are considered, or when the interaction with IFN receptors of different species are dealt with.

It is remarkable to note that several residues, distributed along the sequence, are critical and their replacement can change the biological properties of Type I IFNs.

Mutations of only one of the residues of positions 22, 30, 33, 121, 123, 125, 130, and 131 have been reported to modify (increase or reduction) the biological activity of more than 10-fold with respect to parent protein. In the same cases, as for positions 33 and 121, the kind of the residue is so crucial that even the replacement with amino acids which is commonly believed to be similar, e.g. Arg *vs.* Lys, is not tolerated.

Some positions are determined to confer species specificity. Site-direct mutagenesis of Arg 22 and Arg 33 of human IFNα2c with Lys and His, respectively, produced an analogue which had antiviral activity on human cells 20-fold higher, while it was almost irrelevant for bovine cells [43]. The replacement of Ser 86 of IFNα4 with Cys increased 10-fold the antiviral activity on murine cells, but did not affect the activity on human and bovine cells [44]. Similar results were obtained with the substitution of Arg 121 with Lys in IFNα2 and IFNα4 [44,45]. The replacement of Tyr 123 of IFNα1 with Gly affected the activity on human cells, reducing antiviral potency down to <0.2%, while substitution of the same residue with Lys reduced the activity on human and murine cells 10 times, leaving unalterated the potency on bovine cells [46, 47]. The site-direct mutagenesis of Gln 125 of IFNα2 with Arg or His, respectively, induced antiviral activity higher than 10-fold only on murine cells. A cooperative action should be postulated when the contemporary replacement of positions 121 and 125 are performed, obtaining an analogue of IFNα2 with 400-fold higher antiviral activity, again only on murine cells [45]. The (Ala 30, 32, 33) Hu IFNα2 anlogue acts as antagonist of the parent protein on bovine cells, but not in human cells [39].

It is noteworthy that among the reported data of Table 1 that mutations affecting antiviral activity on bovine cells never occurred, without any corresponding modification of the activity on human cells, inducing one to formulate the hypothesis that the IFN bovine receptor is less sensitive to mutations and can be triggered more easily.

Regarding to the presence of critical residues in relevant positions, it is interesting to note that some properties of IFNα subtypes are strictly correlated to specific amino acidic residues in definite positions, and transferring those to another IFNα subtype has sometimes the effect of transferring the biological properties, too.

The IFNα2 analogue contained in positions 27 and 31 the residues Ser and Met, present only in IFNα1, has biological properties resembling more IFNα1 rather than IFNα2.

Another IFNα2 analogue containing Thr 69 of IFNα1 results in having decreased NK-activity, but that modification was ineffective in changing antiviral and antiproliferative activities [1].

When in hybrid IFN[α8 (1–62), α1(63–92), α8(93–166)], which was active on murine cells, positions 84, 86, 87, and 90 were replaced with those residues present on IFNα8; the hydrid analogue did not interact with murine receptor, resembling that of IFNα8 [48].

Most of the moieties of Type I IFNs have clinical applications. In the case of IFNα, several pharmaceutical preparations are available, some of them based on Cantell's technology, and are constituted by a mixture of IFNα subtypes (LE-IFNα)[49, 50]; others contain IFNα2 subtype produced by DNA recombinant technology (rIFNα) [1]; others are based on the use of lymphoblastoid cells stimulated to produce IFNα with a method similar to that of Cantell, producing a mixture of IFNα subtypes with some differences with respect to the leukocyte IFNα (LY- IFNα) [51, 52].

IFNβ has a clinical applicaton, too. Since it is one molecule, less heterogeneity exists in the pharmaceutical preparations. Three kinds of IFNβ can mainly be distinguished: IFNβ obtained from fibroblast cultures induced with poly I:C, recombinant IFNβ from CHO cells [53], and a mutant anlogue with Ser 17 instead of Cys, obtained in genetically modified *E. coli* [54].

During therapies performed with those products in several cases the onset of antibodies to IFNα (Ab-IFNα) and to IFNβ (Ab-IFNβ) have been detected [55, 56, 57, 58, 59] (see other chapters of this book).

The clinical significance of the onset of Ab-IFN has still not been completely assessed; however, it has been demonstrated in a number of cases that Ab-IFN were responsible for the induction of resistance to IFNα

or IFNβ treatment with a consequent recrudescence of the disease, while IFNα or IFNβ therapy was still continuing [60, 61, 62, 63].

The difference in the induction of Ab-IFNα during the therapy with rIFNα, LY-IFNα, and LE-IFNα [64, 65] and the different cross-reactivity of these Ab-IFNα towards the individual preparations of IFNα [66, 67, 68] suggest that rIFNα, LY-IFNα, and LE-IFNα may show different immunogenicity.

Therefore, the definition of which are the antigenic sites of IFNs, and in particular of IFNα, is of some interest.

From Table 1 it results that several zones of Type I IFNs are available for interactions to mAbs or pAbs. The immunodominant epitopes seem preferentially to contain fragments of Loop AB, BC, and CD. In almost all the cases they are conformational epitopes, confirming that the structure of IFNs is quite compact.

Summarizing the results, Abs to Type I IFNs can be classified in neutralizing Ab (N-Ab), and non-neutralizing Ab (NN-Ab), depending of the ability to abrogate the biological activity of IFNs. NN-Ab-IFNα seem to be directed toward the C-terminal portion of IFNαs [69], while NN-Ab-IFNβ to IFNβ could be direct to internal fragment [70].

N-Ab can be theoretically divided in two subclasses, those that prevent the receptor binding at high affinity [24], and those that forbid the signal transduction [71]. Even minute changes in the primary structures can induce visible changes in antigenic profiles.

Replacement of Arg 23 with Lys, which correspond to the difference between IFNα2b and IFNα2a, modifies the reaction with mAb named 9–1–1 [72]; substitution of Arg 34 of IFNα2c locally modifies the antigenic profile of the molecule [73]; IFNα4a variant with Glu 114 and IFNα4b variant with Val 114 are antigenically distinct in that position [24]. Similar results have been found for the variants a and c of IFNα7, which differ in position 132 for the presence of Met instead of Thr [74].

Mab, named I–4-A, reacts with IFNα4a and IFNα2b, but not with IFNα1 and IFNα14, where the only difference that could explain this selectivity is the residue at position 10 [71].

Since, as reported above, IFNα subtypes differ for residues in critical positions, it commonly occurs that mAbs or pAbs react selectively towards the IFNα subtypes. MAb, designed U1, binds region 5–15 and reacts with IFNα2, but not IFNα1, where the two subtypes differ only in positions 5 and 10 [75].

PAb to region 29–36 binds IFNα1 and IFNα4 but not IFNα2 and IFNβ [75, 76]. The replacement of amino acid residues in positions 113, 114, and 121 of IFNα2 with those of IFNα21, forbids the recognition of mAb-NK–2 to IFNα2, while it does to the parent molecule.

The only common epitope demonstrated up to now between IFNα2 and IFNβ is located in region 132–137 and is recognized by mAb named B6; however, mAb-B6 did not react with IFNα1 [77]. In that fragment the only homology of the sequence between IFNα2 and IFNβ which is not present in IFNα1 is at position 131, where IFNα2 and IFNβ have Lys, while IFNα1 has Thr.

Referring to these data, in the valuation of Ab-IFNα induced during therapeutic treatments with IFNα or IFNβ pharmaceutical preparations the following considerations should be taken in account:

- Ab-IFNα could be directed towards several epitopes with different affinity, therefore different neutralization titers may occur with the same absolute amount of Ab-IFNα.
- Ab-IFN could contain mixtures of N-Ab and NN-Ab that may act sinergistically over the neutralization effect.
- Depending of which epitopes are recognized, only some of the biological activities of Type I IFNs could be selectively inhibited [78].
- Ab-IFN could inhibit Type I IFNs differently depending on the histological origin of cells where they are supposed to act [79].
- The comparison between the neutralizing titers of the sera of different patients can be done properly only in the case where it has been previously demonstrated that N-Ab are directed towards similar epitopes.
- It is exceptional to obtain N-Ab to IFNα mixtures, like those of LE-IFNα, since the same epitopes are not shared by all the IFNα species [74].

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Appendix D

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Antiviral activity of CHO-SS cell-derived human omega interferon and other human interferons against HCV RNA replicons and related viruses

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Abstract

The fully glycosylated human omega interferon produced from CHO-SS cells (glycosylated IFN-ω) has been shown to be well-tolerated in man and to induce a sustained virologic response in patients infected with hepatitis C virus (HCV). We examined the antiviral activity of glycosylated IFN-ω and various human IFNs (IFN-α, -β, -γ and non-glycosylated bacterial (*Escherichia coli*) recombinant IFN-ω (non-glycosylated IFN-ω)) against HCV RNA replicons and several viruses related to HCV. Since none of the IFNs displayed cytotoxicity we compared their activities based on the effective concentration of the IFN that inhibited virus growth by 50% (EC_{50}). Glycosylated IFN-ω was found to be the most potent antiviral agent of all the IFNs tested against bovine viral diarrhea virus (BVDV), yellow fever virus and West Nile virus. With HCV RNA replicons, non-glycosylated IFN-ω was comparable in activity to IFN-α while glycosylated IFN-ω was markedly more potent, indicating that glycosylation has an important effect on its activity. Drug combination analysis of glycosylated IFN-ω + ribavirin (RBV) in BVDV showed a synergy of antiviral effects similar to IFN-α + RBV, as well as a unique antagonism of RBV cytotoxic effects by glycosylated IFN-ω. Transcription factor (TF) profiling indicated that IFN-α or glycosylated IFN-ω treatment upregulated the same 17 TFs. IFN-α and glycosylated IFN-ω also upregulated 9 and 40 additional unique TFs, respectively. The differences in the expression of these TFs were modest, but statistically significantly different for eight of the TFs that were upregulated exclusively by glycosylated IFN-ω. The activation of these additional TFs by glycosylated IFN-ω might contribute to its high potency.

Keywords: Omega interferon; Antiviral; HCV RNA replicons; Drug combination analysis; Transcription factor profiling

1. Introduction

Hepatitis C virus (HCV) infection is the most common cause of chronic hepatitis in the United States and is a major risk factor for the development of liver cirrhosis (Liang et al., 2000). Infection with HCV is currently treated using a combination therapy of pegylated human interferon-α (IFN-α) and ribavirin (RBV), which leads to a sustained virologic response (SVR = absence of detectable HCV RNA in patients 6 months following cessation of therapy) in approximately 55% of all patients (Pearlman, 2004). Other antiviral therapies to treat HCV-infected patients are desperately needed.

The interferons (IFNs) were originally described as biological agents that "interfered" with viral replication (Stark et al., 1998). Human omega interferon (IFN-ω), like other IFNs, is secreted from cells in response to viral infection and it has antiviral, anti-proliferative and immunomodulatory activities (Adolf, 1995). This type I IFN has 62% amino acid identity with alpha interferon (IFNα-2) and 33% amino acid identity with beta interferon (IFN-β), respectively, but it is unrelated to the type II gamma interferon (IFN-γ) (Adolf, 1995). As a distinct IFN,

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IFN-ω, used by itself or in combination with ribavirin or other antiviral therapies, might therefore be beneficial for the treatment of patients who fail to respond to IFN-α or as an additional first-line treatment option.

We have found that the fully glycosylated recombinant human IFN-ω produced from Chinese hamster ovary cells adapted to serum-free growth in suspension culture (CHO-SS) (glycosylated IFN-ω) was well-tolerated in man and that it induced a SVR in patients infected with hepatitis C virus (HCV) genotypes 1–3 (Plauth et al., 2002; Gorbakov et al., 2005). Here we describe the in vitro antiviral activity of glycosylated IFN-ω and other human IFNs against several viruses related to HCV as well as against HCV RNA replicons.

2. Materials and methods

2.1. Antiviral agents

The CHO-SS cell-derived recombinant human IFN-ω is fully-glycosylated and appears to be identical to natural IFN-ω (Buckwold et al., 2006), while the *Escherichia coli*-derived bacterial recombinant human IFN-ω (non-glycosylated IFN-ω; PBL Biomedical Laboratories) is not glycosylated. The other human IFNs utilized in this work were IFN-α (IFNα-2a, Biomedical Laboratories), IFN-β (IFNβ-1a, Biogen, Inc.) and IFN-γ (R&D Systems). RBV was from Sigma.

2.2. Inhibition of viral cytopathic effects assays

The bovine viral diarrhea virus (BVDV) assay in Madin–Darby Bovine kidney cells and the yellow fever virus (YFV) 17D assay in Vero cells were performed as previously described (Buckwold et al., 2003). The West Nile virus (WNV) antiviral evaluations were performed in Vero cells using the WNV isolate NY99-35262-11 from Flamingo (CDC, Ft. Collins, CO) under BSL-3 conditions. Vero cells were grown in Dulbecco's Modified Eagle media (DMEM), 10% fetal bovine serum (FBS), 2% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin-streptomycin. The WNV assay media was the same except that 2% FBS and DMEM without phenol red was used. All cell culture reagents were from Invitrogen. On the day preceding the assay, the cells were trypsinized (1% trypsin–EDTA), pelleted (1200 rpm × 10 min), counted and plated at 2×10^4 well^{-1} in Costar 96-well flat-bottom plates (Corning). The next day compounds were added to the appropriate wells. A pre-titered aliquot of virus was then added to each well in an amount determined to give 85–95% cell killing. After 6 days incubation at 37 °C in a 5% CO_2 incubator, cell viability was determined using CellTiter96 (Promega) with a Wallac Victor microplate reader (Perkin-Elmer). Data analysis was peformed as previously described (Buckwold et al., 2003).

2.3. HCV RNA replicon assays

The HCV RNA replicon antiviral evaluation assay used a Huh7 cell line that contains an HCV RNA replicon called ET (pFK I389 Lucubineo EI NS3-3′ ET = pHCV-ET) that is based on the con1 HCV-1b strain with three cell culture-adaptive mutations and a stable luciferase (Luc) reporter for which expression is directly proportional to HCV RNA replicon RNA levels (Pietschmann et al., 2002). The HCV RNA replicon-containing cell line was kept subconfluent and grown in DMEM, 10% FBS, 1% glutamine, 1% penicillin–streptomycin, 250 μg/ml G418 in a 5% CO_2 incubator at 37 °C, while the assay media was the same except using 5% FBS, DMEM without phenol red, and in the absence of G418. The cells were trypsinized and (5000 well^{-1}) plated in 200 μl of media into the inner wells of two white 96-well plates (Costar) for the Luc-based assay or into one 96-well flat-bottom plate (Costar) for the RNA-based assay. The next day the media was removed and drugs were added in 100 μl assay media to evaluate five half-log concentrations of drug with four replicates each. Ten wells without drugs per plate served as the virus controls (and/or cell controls), 12 wells were for media alone and two wells at each concentration were used for drug color controls. The plates were then returned to the CO_2 incubator for 72 h. In the Luc-based assay, one set of plates was examined for Luc expression as an indirect indication of HCV RNA replicon levels using SteadyGlo reagent (Promega) with a luminometer plate reader (Wallac 1450 Microbeta, Perkin-Elmer), while CytoTox-1 reagent (Promega) was added to the second plate and 560 nM excitation 590 nM emission fluorescence was read with a plate reader (Analyst HT, Molecular Devices) as an indication of cell numbers and cytotoxicity. HCV RNA levels were directly assessed by TaqMan RT-PCR (Martell et al., 1999) with RNA produced in vitro using T7 RNA polymerase (T7 Megascript kit, Ambion) from a pCRII-TOPO (Invitrogen) plasmid containing the cloned TaqMan amplicon (pHCVA) used as the standard. Cellular RNA was extracted from cells using QIAGEN viral RNA 96-well kits. Ribosomal RNA (rRNA) levels determined via TaqMan RT-PCR (Ribosomal RNA control reagents, PE Biosystems) were used as an indication of cell numbers in the RNA-based assay. The average Luc or HCV RNA levels relative to that of the untreated controls, and the background and drug color-corrected fluorescence or rRNA levels relative to that of the untreated controls, was then plotted as a function of drug concentration. The effective drug concentration that reduced HCV RNA replicon levels by 50% (EC_{50}) and 90% (EC_{90}), and the toxic concentration of drug that reduced cell numbers by 50% (TC_{50}) and 90% (TC_{90}) were calculated in dedicated spreadsheets by regression analysis with semilog curve fitting. Selectivity indices (SI = TC/EC) at 50% (SI_{50}) and 90% (SI_{90}) were also calculated.

2.4. Drug combination analysis

The drug combination analyses were performed using the aforementioned model systems, as described previously (Buckwold et al., 2003).

2.5. Transcription factor analysis

HCV RNA replicon-containing ET cells at 75% confluence in T25 flasks were rinsed with PBS and treated with 1,000 IU/ml human IFNα or glycosylated IFN-ω. An untreated flask of cells

served as the control. After 24 h incubation, nuclear extracts were isolated (Nuclear Extraction Kit, Panomics; Jiang et al., 2003) and TranSignal Protein/DNA Arrays I–III (Panomics) were utilized to quantify the activation of 243 TFs, as described previously (Govindarajan et al., 2003; Jiang et al., 2004). The expression levels of each TF was analyzed by comparing the density of each IFN-treated dot on the membrane relative to the untreated control dot using Quantity One v.4.5.2 software (Bio-Rad). The entire experiment was performed three times and the average ±S.D. expression level of each TF whose levels was increased or decreased greater than two-fold relative to the untreated control was determined. We used a Student's *t*-test with $\alpha = 0.05$ to identify those TFs whose expression was statistically significantly different between IFN-α or glycosylated IFN-ω treated samples relative to the untreated control.

3. Results

3.1. Effect of ribavirin and human interferons on viruses related to HCV

Table 1 summarizes the results of the evaluation of a variety of human IFNs and the positive control compound RBV against the cytopathic viruses bovine viral diarrhea virus (BVDV), yellow fever virus (YFV) and West Nile virus (WNV). None of the IFNs displayed cytotoxic effects and inhibitory concentrations of drug that caused the reduction in viable cell numbers by 50% (IC_{50} values) were not reached in any assay. In addition, glycosylated IFN-ω did not display any cytoxicity following seven days at 1000 IU/ml in human peripheral blood mononuclear cells (data not shown). As such, comparisons of drug potency were made based on the antiviral activity using the effective concentrations that reduced viral CPE formation by 50% (EC_{50} values) rather than using selectivity indices ($SI_{50} = IC_{50}/EC_{50}$).

The behavior of IFN-α and RBV against BVDV and YFV was comparable to that observed previously (Buckwold et al., 2003), as were their effects on WNV (Jordan et al., 2000; Anderson and Rahal, 2002; Morrey et al., 2002). IFN-β and IFN-γ were not effective against BVDV and effective concentrations that reduced viral CPE formation by 50% (EC_{50} values) were not reached. In terms of potency (based on EC_{50}), glycosylated IFN-ω was more active than IFN-α against BVDV. All the human IFNs tested were active against YFV with glycosylated IFN-ω and IFN-γ being the most effective, and IFN-α similar to that observed previously (Buckwold et al., 2003), and less effective. Glycosylated IFN-ω was slightly more potent than IFN-γ and more potent than IFN-α against YFV. With WNV, IFN-ω was also more potent than IFN-α.

Table 1
Antiviral activity of human IFNs and RBV against BVDV, YFV and WNV

Virus	Treatment (*n*)	Mean ± S.D.		
		EC_{50}	IC_{50}	SI_{50}[a]
BVDV	IFN-α (9)	29 ± 33 IU/ml	>5000 IU/ml[b]	>170
	IFN-β (2)	NR[c]	>5000 IU/ml[b]	<1
	IFN-γ (2)	NR[c]	>5000 IU/ml[b]	<1
	IFN-ω (9)	1.9 ± 1.2 IU/ml	>5000 IU/ml[b]	>2600
	RBV (9)	10 ± 6.3 μM	32 ± 15 μM	3.2
YFV	IFN-α (3)	150 ± 46 IU/ml	>5000 IU/ml[b]	>33
	IFN-β (1)	140 IU/ml	>5000 IU/ml[b]	>36
	IFN-γ (2)	17 ± 1.6 IU/ml	>5000 IU/ml[b]	>290
	IFN-ω (3)	8.4 ± 5.9 IU/ml	>5000 IU/ml[b]	>600
	RBV (5)	110 ± 56 μM	>410 μM[b]	>3.7
WNV	IFN-α (3)	14 ± 7.9 IU/ml	>1000 IU/ml[b]	>71
	IFN-ω (4)	2.0 ± 2.9 IU/ml	>1000 IU/ml[b]	>500
	RBV (5)	240 ± 71 μM	>410 μMb	>1.7

[a] $SI_{50} = IC_{50}/EC_{50}$.
[b] IC_{50} was not reached.
[c] EC_{50} was not reached.

3.2. Effect of human interferons on HCV RNA replicons

Table 2 shows the results of the comparison of the antiviral activities of IFN-α, glycosylated IFN-ω and non-glycosylated IFN-ω using HCV RNA replicons. The assay was performed in Huh7 cells containing the con1 HCV-1b strain HCV RNA replicon ET (Pietschmann et al., 2002) which has a luciferase (Luc) reporter whose expression is directly proportional to HCV RNA replicon RNA levels. RBV does not show antiviral activity in this assay. The antiviral activities observed were similar using the indirect Luc endpoint or the direct RNA endpoint. None of the IFNs showed any cytotoxic effects and IC_{50} was not reached in any experiment. Based on the EC50 values, glycoslyated IFN-ω was markedly more potent. The antiviral

Table 2
Antiviral activity of human IFNs against HCV RNA replicons

Endpoint	Treatment (*n*)	Mean ± S.D.				
		EC_{50} (IU/ml)	EC_{90} (IU/ml)	IC_{50} and IC_{90} (IU/ml)[a]	SI_{50}[b]	SI_{90}[c]
Luc	IFN-α (14)	0.28 ± 0.088	1.1 ± 0.33	>100[a]	>360	>91
	Glycosylated IFN-ω (10)	0.017 ± 0.019	0.030 ± 0.017	>100[a]	>5900	>3300
	Non-glycosylated IFN-ω (4)	0.13 ± 0.060	0.54 ± 0.23	>100[a]	>770	>190
RNA	IFN-α (8)	0.32 ± 0.092	1.2 ± 0.38	>100[a]	>310	>83
	Glycosylated IFN-ω (5)	0.0047 ± 0.0022	0.017 ± 0.010	>100[a]	>21000	>5900
	Non-glycolsylated IFN-ω (3)	0.20 ± 0.021	0.90 ± 0.087	>100[a]	>500	>110

[a] IC_{50} and IC_{90} were not reached.
[b] $SI_{50} = IC_{50}/EC_{50}$.
[c] $SI_{90} = IC_{90}/EC_{90}$.

activity of non-glycosylated IFN-ω was comparable to IFN-α, as previously described (Cheney et al., 2002; Okuse et al., 2005). This difference in antiviral activity between glycosylated IFN-ω and non-glycosylated IFN-ω indicates that glycosylation has an important effect on its activity.

3.3. Drug combination analysis

A drug combination analysis was then performed using glycosylated IFN-ω + RBV with BVDV. Fig. 1 shows a representative result of this experiment. We observed a synergy of antiviral activity (Fig. 1a and b) between the two drugs (synergy volume = 61 ± 20 IU μg/ml^2 %, n = 3) at physiologically relevant drug concentrations. We also saw a strong antagonism of antiviral effects (antagonism volume = −1100 ± 430 IU μg/ml^2 %, n = 3) between the drugs at high RBV concentrations not observed in vivo (Glue et al., 2000; Larrat et al., 2003; Scott and Perry, 2002; Tsubota et al., 2002), at which RBV exhibits cytotoxicity. In the analysis of the effects of combining glycosylated IFN-ω + RBV on cytotoxicity (Fig. 1c and d), we found a moderate antagonism of the cytotoxic effects of RBV by glycosylated IFN-ω (antagonism volume = −71 ± 38 IU μg/ml^2 %, n = 3) at physiologically relevant drug concentrations. These same effects were also apparent in preliminary experiments when YFV was employed for the drug combination analysis (data not shown).

3.4. Transcription factor analysis in HCV RNA replicons

We next examined the ability of IFN-α and glycosylated IFN-ω to activate cellular transcription factors (TFs) from HCV RNA replicon-containing cells following treatment with 1000 IU/ml human IFN-α or glycosylated IFN-ω. An untreated flask of cells served as the control. After 24 h incubation, nuclear extracts were isolated and TranSignal Protein/DNA Arrays I, II and III (Panomics) were utilized to quantify the activation of 243 TFs, as described previously (Govindarajan et al., 2003; Jiang et al., 2004). The expression levels of each TF was analyzed by comparing the density of each IFN-treated dot on the membrane relative to the untreated control dot. The entire experiment was performed three times and the average ±S.D. expression level of each TF whose levels was increased or decreased greater than two-fold relative to the untreated control was determined (Table 3). No TF showed a significant downregulation of their levels in response to these treatments. The expression of 17 TFs were increased by treatment with either IFN-α or glycosylated IFN-ω. Glycosylated IFN-ω induced the expression of an additional 40 TFs that were not induced by IFN-α; while IFN-α induced the expression of 9 TFs that were not induced by glycosylated IFN-ω.

We used a Student's t-test with $\alpha = 0.05$ to identify those TFs whose expression was statistically significantly different between IFN-α or glycosylated IFN-ω treated samples. Eight TFs which were modestly-induced by glycosylated IFN-ω and not IFN-α were identified: the steroid regulatory element-binding ADD1 (Guan et al., 1995), the gamma IFN promoter repressor AP2/YY1 (Ye et al., 1996), the gamma globulin promoter repressor conserved sequence-binding protein 1 (CSBP; Gumucio et al., 1992), the beta globulin gene promoter erythroid Kruppel-like factor (EKLF; Crossley et al., 1994), the homeotic gene fork head of Drosophila 8 (HFH-8)/hepatocyte nuclear factor 3 (HNF-3)/mouse fork head lung protein (LUN; Miura et al., 1998), HNF-1a (Frain et al., 1989), the interferon consensus sequence binding protein (ICSBP, Driggers et al., 1990) and the lymphocyte-enriched DNA binding protein LyF (Lo et al., 1991).

4. Discussion

In this report we describe the in vitro antiviral activity of the glycosylated IFN-ω and various human IFNs (IFN-α, -β, -γ and non-glycosylated (*E. coli*-derived) IFN-ω (non-glycosylated IFN-ω)) against HCV RNA replicons and several viruses related to HCV. IFNs are not cytotoxic compounds and IC_{50} values were not reached for any IFN in any model system. As such, drug potency comparisons were based on drug EC_{50} values. Glycosylated IFN-ω was more potent than IFN-α against BVDV, YFV and WNV (Table 1). With HCV RNA replicons, non-glycosylated IFN-ω was comparable in activity to IFN-α, while glycosylated IFN-ω was markedly more potent, indicating that glycosylation has an important effect on its activity (Table 2). In each model system that was examined, glycosylated IFN-ω was found to be the most potent antiviral agent of all the IFNs tested.

RBV monotherapy to treat HCV infection is not at all effective while IFN-α only treatment is moderately effective, but the combined use of IFN-α + RBV in patients is much more effective clinically and is currently the standard of care. The reason for this clinical synergy of antiviral activities is not completely understood and both the indirect immune-mediated and direct antiviral effects of RBV may contribute to this success (reviewed in Buckwold, 2004). Glycosylated IFN-ω + RBV also showed a clear synergy of antiviral activity in HCV-infected patients (Gorbakov et al., 2005). We conducted a drug combination analysis of glycosylated IFN-ω + ribavirin (RBV) using BVDV (Fig. 1). A synergy of the antiviral effects of the two drugs was observed which was essentially identical to what we found previously when IFN-α + RBV were examined in this model system (Buckwold et al., 2003). Interestingly, an antagonism of the cytotoxic effects of RBV by glycosylated IFN-ω was observed. This feature was not observed when IFN-α + RBV were tested (Buckwold et al., 2003). Thus the combination of glycosylated IFN-ω + RBV seems favorable both for the synergy of antiviral activities and for the antagonism of RBV drug cytotoxic effects.

Transcription factor (TF) profiling indicated that IFN-α or glycosylated IFN-ω treatment upregulated the same 17 TFs. IFN-α and glycosylated IFN-ω also upregulated 9 and 40 additional unique TFs, respectively. The differences in the expression of these TFs was modest, but statistically significantly different for eight of the TFs that were upregulated exclusively by glycosylated IFN-ω. These IFN-ω-induced TFs were the steroid regulatory element-binding ADD1 (Guan et al., 1995), the gamma IFN promoter repressor AP2/YY1 (Ye et al., 1996), the gamma globulin promoter repressor conserved sequence-binding protein 1 (CSBP; Gumucio et al., 1992), the beta

Table 3
Comparison of transcription factor activation in HCV RNA replicon-containing Huh7 ET Cells by glycosylated IFN-ω and IFN-α

Activation by	Transcription factor	Description	Glycosylated IFN-ω/control[a]	IFN-α/control[a]
Both glycosylated IFN-ω and IFN-α	HFH-3	Forkhead box I1	3.59 ± 3.96	3.13 ± 3.39
	RREB (sequence 2)[b]	Ras-responsive transcription element	3.47 ± 3.36	3.94 ± 4.61
	HBS/xbp1	HIF binding sequence (rat, as human xbp-1)	3.21 ± 3.23	3.61 ± 4.30
	PARP	Poly(ADP-ribose) synthetase/polymerase	3.16 ± 3.52	2.52 ± 5.19
	MSP1	Amyloid precursor protein (APP) regulatory element	2.85 ± 2.59	2.08 ± 3.74
	VDR (DR-3)	VDR: Vitamin D (1,25-dihydroxyvitamin D3) receptor	2.79 ± 1.68	2.43 ± 1.06
	MEF-2 (sequence 1)[b]	Myelin gene expression factor	2.66 ± 2.13	2.15 ± 4.16
	Stat4	Signal transducer and activator of transcription 4	2.60 ± 1.36	2.06 ± 1.32
	PPUR (sequence 1)[b]	Purine-rich sequences binding sequence	2.54 ± 1.15	2.15 ± 1.20
	MT-Box	Tentative new binding domain	2.30 ± 2.19	2.52 ± 1.92
	HIF-1	Hypoxia-inducible factor 1	2.29 ± 2.42	2.61 ± 2.48
	RXR (DR1)	Retinoic acid X receptor	2.29 ± 1.18	2.45 ± 1.21
	TR (DR4)	Thyroid hormone receptor	2.15 ± 1.20	2.13 ± 1.17
	MyoD	Myogenic factor D	2.15 ± 1.20	2.01 ± 1.62
	NF-κB	Nuclear factor of kappa light enhancer in B-cells	2.11 ± 1.42	2.25 ± 1.81
	Pax4	Paired box gene 4	2.08 ± 1.44	2.39 ± 2.26
	USF-1	Upstream transcription factor	2.01 ± 1.29	2.26 ± 1.80
Glycosylated IFN-ω alone	c-Rel	NF-κB p75 kDa protein	3.85 ± 4.56	1.19 ± 0.18
	E4F, ATF	E4F transcription factor 1	2.96 ± 3.11	1.38 ± 0.23
	GATA-4	GATA binding protein 4	2.88 ± 2.42	1.64 ± 1.13
	E4BP4	Nuclear factor, interleukin 3 regulated	2.81 ± 1.78	1.56 ± 0.61
	CP1, CTF, CBTF	CCAAT-box-binding transcription factor	2.73 ± 1.09	1.62 ± 0.87
	HOXD 9,10	Homeo box D9, D10	2.73 ± 0.90	1.56 ± 0.83
	LyF	LyF binding site	2.65 ± 0.42	1.55 ± 0.60
	LyF-1 (sequence 1)[b]	LyF-1 binding site	2.64 ± 0.77	1.73 ± 0.67
	ATF/CRE	ATF/CRE binding site	2.64 ± 0.97	1.27 ± 0.67
	HNF-1A	Hepatocyte nuclear factor	2.54 ± 0.59	1.18 ± 0.24
	E47	E2A enhancer binding factors E12/E47	2.52 ± 1.61	1.22 ± 0.60
	HFH-8, HNF-3, LUN	A new mouse forkhead gene named LUN	2.52 ± 0.56	1.03 ± 0.15
	ZIC	A DNA binding domain on the EBV BZLF1 promoter	2.46 ± 2.19	1.52 ± 1.29
	Pax3	Paired box gene 3	2.37 ± 2.08	1.80 ± 3.01
	RSRFC4	MADS box transcription enhancer factor 2	2.37 ± 0.49	1.54 ± 1.12
	CSBP	Conserved sequence-binding protein	2.34 ± 0.79	1.13 ± 0.40
	ATF2	ATF2	2.30 ± 1.88	1.86 ± 1.49
	MTB-Zf	*Cis* regulatory element (MTE) binding protein	2.26 ± 0.90	1.70 ± 0.73
	CREB	cAMP responsive element binding protein 1	2.26 ± 0.84	1.48 ± 0.65
	NF-Y	Nuclear Y box factor	2.23 ± 1.07	1.92 ± 1.14
	PEBP2	Polyoma enhancer binding protein	2.20 ± 0.80	1.98 ± 0.86
	AP-2	Activating enhancer binding protein	2.19 ± 0.97	1.87 ± 0.08
	MyT1	Myelin transcription factor I	2.19 ± 0.78	1.28 ± 0.28
	ADR1	ADH regulatory gene-1 binding element	2.19 ± 1.79	1.11 ± 0.22
	MAZ	MYC-associated zinc finger protein	2.18 ± 0.98	1.46 ± 1.30
	CETP/CRE	Cholesterol esterase transfer protein/response element	2.16 ± 1.67	1.13 ± 0.08
	EVI-1	Ecotropic viral integration site 1 (zinc finger oncogene)	2.13 ± 1.93	1.31 ± 0.74
	Antioxidant RE	Antioxidant responsive element	2.13 ± 1.24	1.20 ± 0.52
	AhR/Arnt	Aryl hydrocarbon receptor/nuclear translocator binding element	2.13 ± 1.39	1.11 ± 0.39
	LCR-F1	Nuclear factor (erythroid-derived 2)-like 1	2.11 ± 1.35	1.77 ± 1.16
	AIC, CBF	ApoA-I gene promoter c region, CCAAT-binding factor	2.09 ± 0.96	1.02 ± 0.13
	EKLF (sequence 1)[b]	Erythroid Kruppel-like factor gene	2.08 ± 0.36	1.14 ± 0.12
	ADD1	Sterol regulatory element binding transcription factor	2.07 ± 0.68	1.08 ± 0.37
	LR1	A 106-kDa sequence-specific DNA-binding protein	2.06 ± 0.81	1.37 ± 0.54
	Smad3/4	MADH3/4, mothers against decapentaplegic homolog 3/4	2.05 ± 0.86	1.70 ± 0.81
	NF-E1 (YY1)	YY1 transcription factor	2.04 ± 0.88	1.83 ± 0.75
	CACC	CACC binding protein	2.04 ± 1.33	1.31 ± 0.69
	Brn-3	POU4F1: POU domain, class 4, transcription factor 1	2.02 ± 0.78	1.71 ± 0.58
	ICSBP	Interferon consensus sequence binding protein	2.02 ± 0.69	1.06 ± 0.22
	AP2, YY1	Activating enhancer binding protein 2-like YY1 site	2.02 ± 0.44	0.85 ± 0.16
IFN-α alone	HBS + HAS	Hypoxia-inducible factor 1 binding sequence/ancillary sequence	1.90 ± 1.06	2.51 ± 2.10
	Pax6	Paired box gene 6	1.88 ± 0.29	2.21 ± 0.79

Table 3 (*Continued*)

Activation by	Transcription factor	Description	Glycosylated IFN-ω/control[a]	IFN-α/control[a]
	MUSF1	Amyloid precursor protein regulatory element without USF site	1.84 ± 0.53	2.32 ± 0.87
	MZF1	Zinc finger protein 42 (myeloid-specific retinoic acid-responsive)	1.70 ± 0.42	2.61 ± 1.86
	ISRE (sequence 2)[b]	Interferon-α stimulated response element	1.69 ± 1.24	2.32 ± 1.89
	NZF-3	Neural zinc finger factor 3	1.51 ± 0.30	2.29 ± 1.69
	XRE	Xenobiotic response element	1.44 ± 0.49	2.01 ± 1.65
	RREB (sequence 1)[b]	Ras-responsive transcription element	1.38 ± 0.82	2.50 ± 0.37
	XBP-1	X-box binding protein 1	1.24 ± 0.45	2.05 ± 1.55

[a] Mean ± S.D.
[b] Sequence 1 or 2 as designated by Panomics.

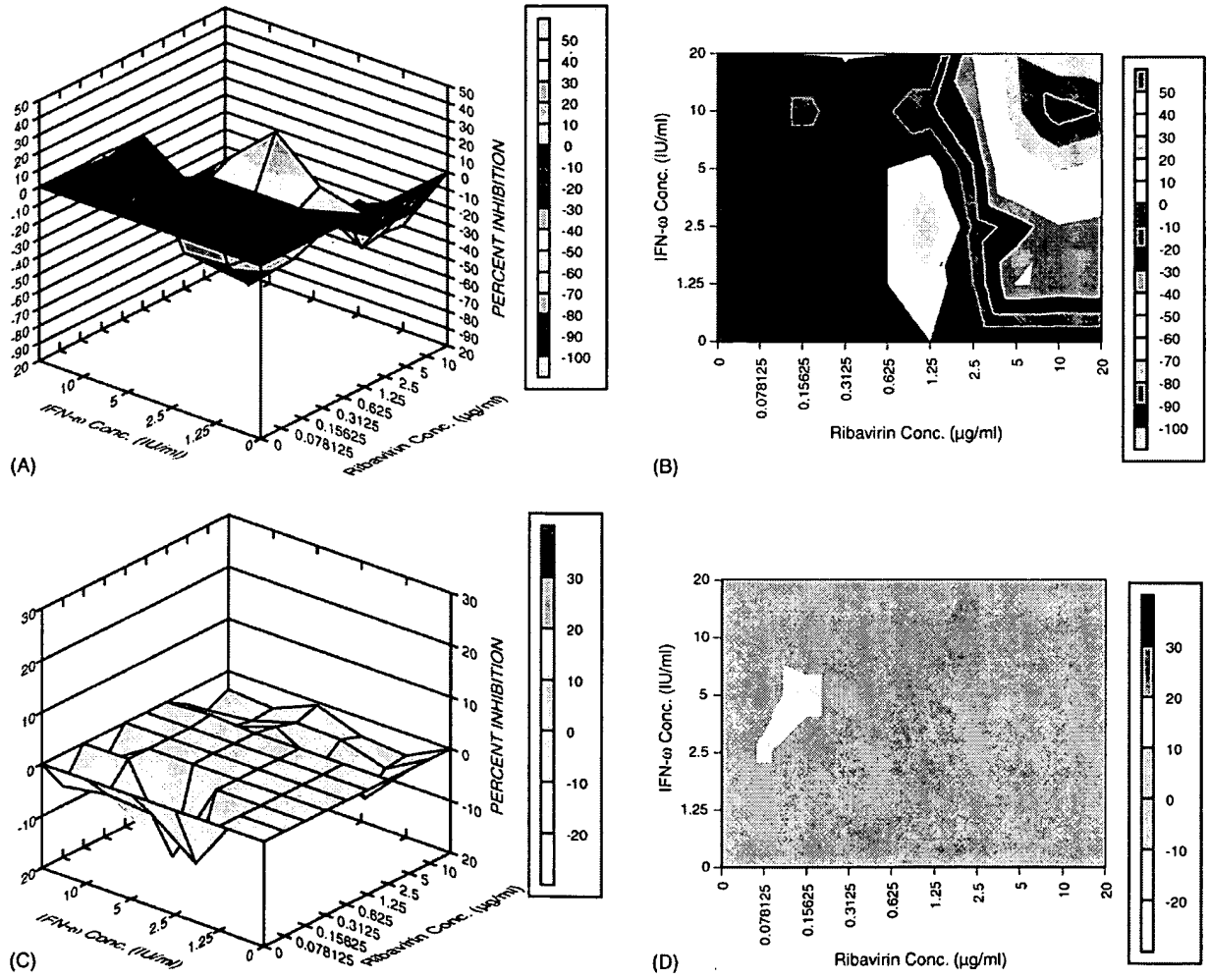

Fig. 1. Drug combination analysis of glycosylated IFN-ω + RBV using BVDV in MDBK cells. The calculated additive interactions between the drugs were subtracted from the experimentally determined values based on mean background- and drug color-corrected data to reveal the regions and corresponding drug concentrations at which synergistic or antagonistic interactions affecting antiviral activity and drug cytotoxicity occurred. Peaks of statistically significant synergy or antagonism that deviate significantly from the expected additive drug interactions derived from 95% confidence interval data are shown in the difference plots of the interactions between IFN-ω and RBV (A and C) and in the corresponding contour plots (B and D). The colors indicate the level of synergy or antagonism with the corresponding peak volumes found at each drug concentration indicated in the sidebar. The effects of the combination of IFN-ω and RBV on antiviral activity is shown in panels A and B, while the effects of the drug combination on cytotoxicity in MDBK cells are shown in panels C and D. The experiment was repeated three times with essentially identical results observed.

globulin gene promoter erythroid Kruppel-like factor (EKLF; Crossley et al., 1994), the homeotic gene fork head of Drosophila 8 (HFH-8)/hepatocyte nuclear factor 3 (HNF-3)/mouse fork head lung protein (LUN; Miura et al., 1998), HNF-1a (Frain et al., 1989), the interferon consensus sequence binding protein (ICSBP, Driggers et al., 1990) and the lymphocyte-enriched DNA binding protein LyF (Lo et al., 1991). The molecular basis of this differential regulation of host cell TFs in response to IFN-α and glycosylated IFN-ω treatment is uncertain both due to the modest nature of the activation observed and since it is unclear from these experiments whether the observed effects are transcriptional or post-transcriptional in nature. However, both IFN-α and glycosylated IFN-ω are type I IFNs that affect cells by engaging what appears to be the same cellular receptors. It is possible that structural differences may cause them to interact with their receptors in different ways since such differences are thought to explain in part, some of the significant differences observed in the clinical and biological activities of the other type I interferons (Karpusas et al., 1997; Walter, 1997). Since glycosylated IFN-ω induced the activation of several other TFs that were not induced by treatment with IFN-α, this may be in part responsible for its high potency.

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Appendix E

Digestive Disease Week 2007 Meeting
Poster number M1798
Poster Session Monday, May 21, 2007

Poster number M1798
Poster Session Monday, May 21, 2007

Phase 2 Study of Omega Inte in Interferon-naïve Subj

V. Novozhenov[1], N. Zakharova[2], E. Vinogradova[2], I. Nikitin[3], V. Gorbakov[4], A. Yak

Introduction

Hepatitis C virus represents a global health issue, affecting more than 170 million people worldwide. The current standard treatment, pegylated interferon-alpha and ribavirin, fails to induce a sustained viral response in approximately 50% of treated patients. Poor compliance and frequent treatment discontinuations represent significant obstacles to successful treatment. This phase 2 study is designed to evaluate the safety and antiviral activity of omega interferon in combination with ribavirin for the specific purpose of selecting an appropriate dose for a subsequent study of omega interferon continuous administration with the DUROS® continuous delivery device.

Omega Interferon

- Recombinant human type-1 Interferon
- 70% homology with alpha interferons
- Derived from CHO cells
- Fully glycosylated
- In vitro potency ≥ alpha interferon in BVDV, WNV, YFV and HCV replicons[1]

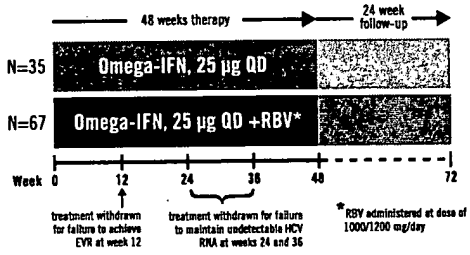
1. Buchwald, V., Antiviral Research: 73 (2007) pp. 118-125

Aim

- To evaluate the comparative activity and safety of omega interferon daily injections with or without ribavirin
- To determine a safe and active starting dose for a planned Omega DUROS continuous delivery device phase 1b dose-escalating study

Methods

- 102 genotype-1 interferon-naïve subjects
- Randomized, open-label
  - 1:2 randomization of the two study arms
- Endpoints: EVR & SVR



- Dose reductions
  - Omega interferon 25 µg/day to 10 µg/day for ANC <750/mm³
  - Ribavirin 1000/1200 mg/day to 600 mg/day for Hgb<10g/dL

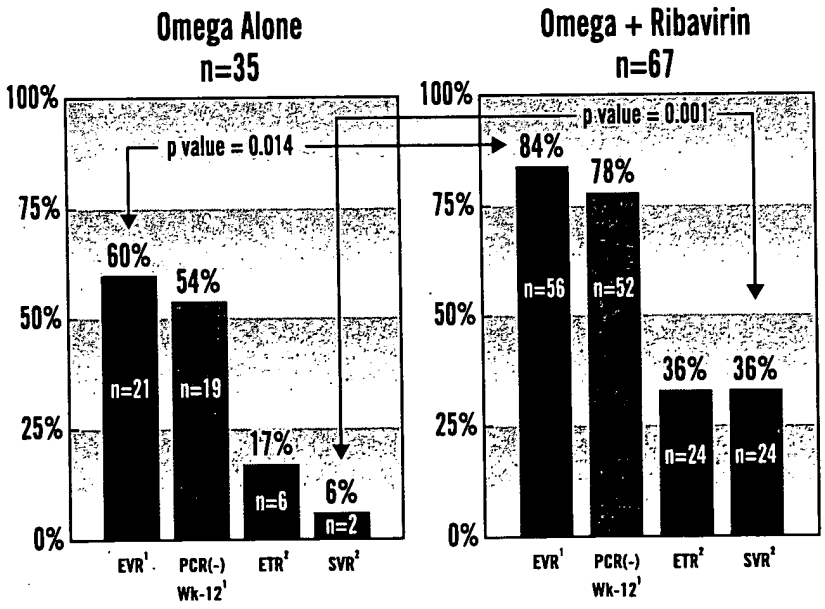
Inclusion/Exclusion Criteria

- **Inclusion**
  HCV genotype-1
  Detectable HCV RNA
  ALT > ULN
  Informed consent
- **Exclusion**
  Other causes of chronic liver disease
  Lab values: Hgb < 12g/dL Platelet < 100,000/mm³
  WBC < 3000/mm³ ANC < 1500/mm³
  Any prior interferon treatment
  HIV-positive

Patient Demographics

Parameter		Omega Interferon Alone (N = 35) n (%)	Omega Interferon Plus Ribavirin (N = 67) n (%)
Gender	Female	12 (34.3%)	28 (41.8%)
Ethnicity	White	35 (100%)	67 (100%)
Age	Mean Range	33.4 19-56	35.4 19-62
Weight	≤ 75 kg	13 (37.1%)	36 (53.7%)
Baseline HCV RNA (IU/mL x 1000)	Mean Range	3688 163-18100	3276 209-14157

HCV RNA Response



1. LOQ 600 IU/mL 2. LOD 50 IU/mL

All patients achieving SVR also experienced ALT normalization

feron Alone or in Com
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lev[5], S. Pak[6], V. Rafalski[7], P. Bogomolov[8], T. Aless

Subject Discontinuations & SAEs

	Omega Interferon Alone (N = 35) n (%)	Omega Interferon Plus Ribavirin (N = 67) n (%)
Completed the study	35/35 (100%)	63/67 (94.0%)
Subjects discontinued	0/35 (0.0%)	4/67 (6.0%)
Adverse events	0	2 (3.0%)
Withdrew consent	0	2 (3.0%)
Serious adverse events	3 (8.6%)	1 (1.5%)
Cellulitis	1 (2.9%)	0 (0%)
Ulna fracture	1 (2.9%)	0 (0%)
Fibroadenoma of breast	0 (0%)	1 (1.5%)
Pneumonia	1 (2.9%)	0 (0%)

Dose Reductions

Drug Reduced	Omega Interferon Alone (N = 35) n (%)	Omega Interferon Plus Ribavirin (N = 67) n (%)
Omega interferon only	2 (5.7%)	5 (7.5%)
Ribavirin only	0 (0%)	12 (17.9%)
Omega interferon and ribavirin	0 (0%)	8 (11.9%)

Clinical Laboratory Abnormalities NCI CTC Grade 3 & 4

	Omega Interferon Alone (N = 35) n (%)	Omega Interferon Plus Ribavirin (N = 67) n (%)
All	17 (48.6%)	36 (53.7%)
Neutrophils decrease	8 (22.9%)	27 (40.3%)
ALT increase	11 (31.4%)	9 (13.4%)
WBC decrease	0 (0%)	9 (13.4%)
Lymphocyte decrease	0 (0%)	5 (7.5%)
Sodium decrease	2 (5.7%)	3 (4.5%)
Indirect bilirubin increase	0 (0%)	3 (4.5%)
Potassium increase	0 (0%)	2 (3.0%)
Total bilirubin increase	0 (0%)	2 (3.0%)
AST Increase	1 (2.9%)	0 (0%)
Potassium decrease	1 (2.9%)	0 (0%)

bination with Ribavirin hronic Hepatitis C

[9], D. Blanchett[9], W. Lang[9], P. Langecker[9], J. McNally[9], B. Bacon[10]

Adverse Events

MedDRA Preferred Term includes grade 1-4	Omega Interferon Alone (N = 35) n (%)	Omega Interferon Plus Ribavirin (N = 67) n (%)
Influenza-like illness	15 (42.9%)	32 (47.8%)
Neutropenia	12 (34.3%)	29 (43.3%)
Asthenia	13 (37.1%)	19 (28.4%)
Pyrexia	8 (22.9%)	20 (29.9%)
Leukopenia	1 (2.9%)	19 (28.4%)
Fatigue	4 (11.4%)	13 (19.4%)
Anemia	0 (0.0%)	14 (20.9%)
Headache	2 (5.7%)	10 (14.9%)
Blood bilirubin increased	1 (2.9%)	10 (14.9%)
Depression	0 (0.0%)	2 (3.0%)

Hematology Lab Values

	Omega Interferon Alone (N = 35) n (%)	Omega Interferon Plus Ribavirin (N = 67) n (%)
ANC < 750 x 10^3 µL	3 (8.6%)	11 (16.4%)
ANC < 500 x 10^3 µL	0 (0.0%)	1 (1.5%)
HGB < 12 g/dL	9 (25.7%)	39 (58.2%)
HGB < 10 g/dL	1 (2.9%)	5 (7.5%)
Platelets < 50,000/mm^3	0 (0.0%)	0 (0.0%)

Conclusions

- Omega interferon and ribavirin resulted in EVR and SVR rates similar to those reported with the combination of alpha interferon and ribavirin in interferon-naïve HCV genotype-1 patients.
- EVR and SVR rates achieved with omega interferon plus ribavirin were superior to those achieved with omega interferon alone.
- Omega interferon given alone or in combination with ribavirin was well tolerated with few patients requiring dose reductions or treatment discontinuation.
- 25 µg/day is an appropriate starting dose to evaluate continuous delivery Omega DUROS therapy in combination with ribavirin in future studies.

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Omega DUROS Phase 1b: Study Design

Dose escalation in genotype-1 patients relapsing after prior Peg IFN + RBV